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(71) Applicant: ARCH DEVELOPMENT CORPORATION [US/US]; 1101 East 58th Street, Chicago, IL 60637 (US).

(72) Inventors: BLUESTONE, Jeffrey, A.; 5421 Kenwood Avenue, Chicago, IL 60637 (US). LENSCHOW, Deborah, J.; 5455 South Blackstone Avenue, Chicago, IL 60615 (US).

(74) Agent: PARKER, David, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: B7-2: CTLA4/CD28 COUNTER RECEPTOR

(57) Abstract

The invention relates generally to compositions of and methods for obtaining and using a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. The invention relates as well to the polynucleotides sequences encoding those polypeptides, the recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors, and recombinant polypeptides. The invention includes as well, methods for using the isolated, recombinant polypeptides in assays designed to select and improve substances capable of altering T cell activation for use in diagnostic, drug design and therapeutic applications.

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B7-2: CTLA4/CD28 COUNTER RECEPTOR.

Field of the Invention

This invention relates generally to compositions of and methods for regulating T cell activation. The invention relates more particulary to DNA sequences that encode polypeptides that regulate T cell activation, the recombinant vectors carrying those sequences, the recombinant host cells including either the sequences, vectors, or polypeptides which regulate T cell activation. The invention includes as well methods for using isolated, recombinant polypeptides in assays designed to select and improve among candidate substances that affect T cell activation. The invention also includes polypeptides and polynucleotides for use in diagnostic, drug design and therapeutic applications.

Background of the Invention

The immune system can promptly respond to millions of foreign antigens. One of its responses to a foreign antigen is the proliferation of a class of lymphocytes which specifically recognizes the antigen. The proliferation of the T cells of the immune system is part of a multifaceted response of T lymphocytes known as T cell activation. One of the cell surface proteins of a T cell is a T cell receptor. Antigen-specific T cell activation depends on T cell receptor (TCR) interaction with peptide/major histocompatibility complex (MHC) in conjunction with co-stimulatory signals mediated by accessory molecules. T cell clones encountering nominal antigen/MHC complexes in the absence of appropriate co-stimulation are functionally inactivated. This induced nonresponsiveness, termed "T cell anergy", is defined by the failure of T cells to proliferate and produce lymphokines, such as IL-2. Several investigators have suggested that the interaction of the CD28 molecule on the T cell with a ligand, B7, on the antigen-presenting cell (APC) is best characterized among the many cell surface

receptor/ligand pairs in delivering this costimulatory activity. Information concerning the role of ligand interaction with CD28 can be found in PCT Application Number PCT/US89/05304, published as International Publication Number WO90/05541, the disclosure of which is incorporated herein by reference.

CD28 is a cell surface glycoprotein constitutively expressed on most T cells, which has been recently shown to interact with B7, which is expressed on dendritic cells, macrophages, and activated B and T cells. Blocking the binding of CD28 on T cells to its ligand, B7/BB-1, during TCR engagement, results in T cell anergy.

Antibodies directed against CD28 can prevent this induced unresponsiveness by stimulating T cells through a distinct signalling pathway. Signals delivered via CD28 are independent of TCR signalling, insensitive to cyclosporin A, and control the production of IL-2 by both transcriptional and post-transcriptional regulation.

Another ligand for B7 is CTLA4, a cell surface protein which has a high degree of similarity to CD28 and is expressed on T cells following activation. Human CTLA4Ig, a soluble form of this protein, formed by fusing the extracellular domain of the human CTLA4 gene to a human IgG1 Fc domain, binds to B7 with a 10-20 fold higher affinity than CD28, and inhibits a variety of immunological responses. We have shown that blocking the CD28 signalling pathway with CTLA4Ig blocks allo- and xenoantigen responses *in vitro*, prevents xenogeneic transplant rejection, and induces long term, antigen-specific unresponsiveness *in vivo*.

This invention discloses a new ligand, B7-2, which binds to CTLA4Ig. B7-2 is constitutively expressed on dendritic cells and rapidly upregulated on activated B cells. This novel ligand, B7-2, plays an important functional role in stimulating primary T cells responses *in vitro*.

Brief Summary of the Invention

In one aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CTLA4Ig, or CD28, or homologous proteins, and regulates T cell activation. In a preferred embodiment, a polynucleotide of the present invention is a DNA molecule. More preferably, a polynucleotide of the present invention encodes a polypeptide designated B7-2.

Yet another aspect of the present invention contemplates an isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases of a polynucleotide that encodes for B7-2 wherein the polynucleotide hybridizes to a polynucleotide that encodes an for B7-2. Preferably, the isolated and purified polynucleotide comprises a base sequence that is identical or complementary to a segment of at least 25 to 70 contiguous bases of a polynucleotide that encodes for B7-2. For example, the polynucleotide of the invention can comprise a segment of bases identical or complementary to 40 or 55 contiguous bases of a polynucleotide that encodes for B7-2.

In another embodiment, the present invention contemplates an isolated and purified polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation of the invention is a recombinant polypeptide. More preferably, a polypeptide of the present invention is B7-2.

In an alternative embodiment, the present invention provides an expression vector comprising a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, an expression vector of the present invention

comprises a polynucleotide that encodes a B7-2 polypeptide. Even more preferably, an expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More preferably still, an expression vector of the invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter, and the expression vector further comprises a polyadenylation signal that is positioned 3′ of the carboxy-terminal amino acid and within a transcriptional unit of the encoded polypeptide.

In yet another embodiment, the present invention provides a recombinant host cell transfected with a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, a recombinant host cell of the present invention is transfected with a polynucleotide that encodes for B7-2. Preferably, a host cell of the invention is a eukaryotic host cell. Still more preferably, a recombinant host cell of the present invention is a yeast cell. Alternatively, a recombinant host cell of the invention is a COS-1 cell.

In another aspect, a recombinant host cell of the present invention is a prokaryotic host cell. Preferably, a recombinant host cell of the invention is a bacterial cell of the DH5α strain of *Escherichia coli*. More preferably, a recombinant host cell comprises a polynucleotide under the transcriptional control of regulatory signals functional in the recombinant host cell, wherein the regulatory signals appropriately control expression of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation in a manner to enable all necessary transcriptional and post-transcriptional modification.

In yet another embodiment, the present invention contemplates a process of preparing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising

transfecting a cell with polynucleotide that encodes a polypeptide of the present invention to produce a transformed host cell; and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide. Preferably, the transformed host cell is a eukaryotic cell. More preferably still, the eukaryotic cell is a COS-1 cell. Alternatively, the host cell is a prokaryotic cell. More preferably, the prokaryotic cell is a bacterial cell of the DH5α strain of *Escherichia coli*. Even more preferably, a polynucleotide transfected into the transformed cell comprises the nucleotide base sequence that encodes for B7-2.

In still another embodiment, the present invention provides an antibody immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, an antibody of the invention is a monoclonal antibody. Even more preferably, the monoclonal antibody is immunoreactive with B7-2.

In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising the steps of (a) transfecting a recombinant host cell with a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation; (b) culturing the host cell under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing the antibody to the polypeptide. Preferably, the host cell is transfected with the polynucleotide of that encodes for B7-2. Even more preferably, the present invention provides an antibody prepared according to the process described above.

Alternatively, the present invention provides a process of detecting a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises immunoreacting the polypeptide with an antibody prepared according to

the process described above to form an antibody-polypeptide conjugate, and detecting the conjugate.

In yet another embodiment, the present invention contemplates a process of detecting a messenger RNA transcript that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises (a) hybridizing the messenger RNA transcript with a polynucleotide sequence that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation to form a duplex; and (b) detecting the duplex. Alternatively, the present invention provides a process of detecting a DNA molecule that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises (a) hybridizing DNA molecules with a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation to form a duplex; and (b) detecting the duplex.

In another aspect, the present invention contemplates a diagnostic assay kit for detecting the presence of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation in a biological sample, where the kit comprises a first container containing a first antibody capable of immunoreacting with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, with the first antibody present in an amount sufficient to perform at least one assay. Preferably, an assay kit of the invention further comprises a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in an assay kit of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

In an alternative aspect, the present invention provides a diagnostic assay kit for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases a polynucleotide that encodes for B7-2.

In another embodiment, the present invention contemplates a diagnostic assay kit for detecting the presence, in a biological sample, of an antibody immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, the kit comprising a first container containing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation that immunoreacts with the antibody, with the polypeptide present in an amount sufficient to perform at least one assay.

In yet another aspect, the present invention contemplates a process of screening substances for their ability to interact with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising the steps of providing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, and testing the ability of selected substances to interact with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

Brief Description of the Drawings

Figure 1A-L. Anti-B7 antibodies have equal or higher affinity for B7 than does hCTLA4Ig. CHO cells transfected with murine B7 were incubated with the indicated concentration of unlabelled blocking antibodies and stained, as described in methods, with the following antibodies at optimal staining concentrations:

biotinylated hCTLA4Ig, biotinylated anti-mB7 (16-10A1), or FITC-coupled anti-mB7 (1G10). The biotinylated reagents were then developed with PE-SA, and analyzed by flow cytommetry. In control experiments, untransfected CHO cells were not stained by any of the three reagents. The top panel of each staining condition shows the profile for unstained cells (–) using the appropriate control Ig. Histograms are displayed with fluorescent intensity on the x axis and cell number on the y axis.

Figure 2A-L. The CTLA4 ligand is expressed on splenic dendritic cells and on activated B cells. Splenic dendritic cells were isolated as described. Spleen cells were stimulated with LPS (10 μg/ml) for 3 days, harvested, viable cells recovered by Ficoll-hypaque separation, and stained with anti-B7 mAbs and CTLA4Ig. Unstimulated and LPS blasted spleen cells were incubated with 2.4G2 to block FcR binding and then incubated with 30 μg of the indicated blocking antibodies. Cells were then stained for surface expression with FITC coupled B220 mAb and biotinylated hCTAL4Ig or anti-B7 (16-10A1) and counter-stained with PE-SA. Dendritic cells were incubated with N418 and counter-stained with a FITC-conjugated anti-hamster IgG, washed and then incubated with the appropriate blocking antibody. The dendritic cells were then stained with either biotinylated hCTLA4Ig or anti-B7 (16-10A1) and developed by PE-SA. Cells were subjected to two color flow cytometry, and data was displayed as contour plots with green (FITC) fluorescence on the x axis and orange (PE) fluorescence on the y axis. The percent positive cells are indicated.

Figure 3. Differential regulation of the B7-2 and B7 expression on activated splenic B cells.

A.) Splenic B cells from C57BL/6 mice were incubated with LPS (10 μg/ml) (circles) or Con A (2.5 μg/ml) (triangles) for the indicated amount of time. Cells were then harvested and stained as described in methods. The B7-2 (solid lines) was measured as the net amount of mean fluorescence observed with hCTLA4Ig-bio in the presence of uncoupled anti-B7 (16-10A1) mAb. B7

expression was measured by staining with anti-B7 mAb (16-10A1) (dotted lines). All staining reagents and FACSan settings were kept constant so that the mean fluorescence intensity could be compared between time points. Incubation of cells in media for the duration of the time course did not induce up-regulation of either B7 or B7-2.

B.) Spleens were T-depleted as described in methods with anti-Thy1 and complement treatment. Cells were then incubated with Con A (2.5 μg/ml) or LPS (10 μg/ml) for 24 hours and then harvested. Cells were stained as described above and analyzed by two color flow cytometry with anti-B220-FITC and hCTLA4Ig or anti-B7-BIO as described in figure 2.

Figure 4. Only hCTLA4Ig inhibits T cell activation to whole spleen, while both hCTLA4Ig and anti-B7 block T cell activation to a B7-transfectant.

A.) The TH1 clone, PGL2 (5×10^4) was incubated with 25 μg/ml peptide (DOT) and 1×10^5 mitomycin C-treated EL-4 cells transfected with I-A^d and B7 (ELAD/B7). Blocking antibodies, at the indicated concentrations, were incubated with the stimulator population for 15 minutes prior to the addition of the TH1 clone, PGL2. The untreated group represents PGL2 proliferation to ELAD/B7 and antigen without additional blocking antibodies. No proliferation was observed using non-transfected EL-4 cells.

B.) PGL2 (5×10^4) was incubated with 2.5×10^5 irradiated BALB/c spleen cells pulsed with 25 μg/ml peptide (DOT). Blocking antibodies were added as described above. T cell activation was measured by [^{3}H]-thymidine incorporation into DNA and is expressed as the mean CPM of triplicate wells. SE were $<10\%$.

Figure 5. Dendritic cells expressing the B7-2 are important in initiating proliferation in a MLR. Splenic dendritic cells from C57BL/6 mice were enriched as described in methods. Dendritic cell purity ranged in various experiments from 40 to 90% using N418 as a marker. DBA LN responders (6×10^5 cells/well)

were incubated with either whole spleen at (5×10^5 cells/well) or with enriched dendritic cells at (5×10^4). All stimulator cell populations were irradiated with 2000 rads. The open bars represent the response in the absence of blocking antibodies. All other groups were incubated with the indicated blocking reagent at 100 μg/ml. T cell activation was measured by 3H-thymidine uptake into DNA and is expressed as the mean cpm of triplicate wells for each condition. SE were <10%.

Detailed Description of the Invention

I. The Invention

Effective T cell activation requires antigen/MHC engagement by the T cell receptor complex in concert with one or more co-stimulatory molecules. Recent studies have suggested that the B7 molecule, expressed on most antigen presenting cells (APC), functions as a co-stimulatory molecule through its interaction with CD28 on T cells. Blocking CD28/B7 interactions with CTLA4Ig (a soluble homologue of CD28 that binds B7), inhibits T cell activation in vitro and induces T cell clones into a state of unresponsiveness. In this invention, we demonstrate that another molecule(s), termed B7-2, can provide a co-stimulatory signal to native T cells responding to alloantigen. As used herein, binding signifies specific binding. Although both B7 and B7-2 bind to CTLA4Ig and are expressed constitutively on dendritic cells, these molecules are differentially regulated on B cells. B7-2 is upregulated by lipopolysaccharide in less than six hours, and is maximally expressed on the majority of B cells by 24 hours. In contrast, B7 is only detected on a subset of activated B cells late (48 hours) after stimulation. In addition, Concanavalin A (Con A) directly induces B7-2 expression on B cells, but does not induce B7 expression. Finally, although both anti-B7 mAbs and CTLA4Ig blocked T cell activation to antigen-expressing B7 transfectants, only CTLA4Ig had any significant inhibitory effect on T cell activation to antigens expressed on natural APCs, such as dendritic cells. Thus B7 is not the only

co-stimulatory molecule capable of initiating T cell responses since a novel ligand, B7-2, can provide a necessary second signal for T cell activation.

The regulation of T cell activation is quite complex, including not only antigen specific recognition by the T cell receptor (TCR), but also a cascade of adhesion and costimulatory events. These events are directed by various receptor-ligand pairs found on T cells and APCs, that include LFA-1/ICAM-1 & ICAM-2, VLA-4/VCAM-1, HSA/?, CD40/gp39, and CD28/B7. These receptor/ligand pairs have been shown to increase cellular adhesion between the T cell and APC, and/or to provide necessary signals to co-stimulate both T and B cells. Another characteristic of these cell surface molecule interactions is that many of the receptors can bind to more than one ligand. This reflects the ability of the various receptor/ ligand interactions to provide signals which differentially regulate T and B cell responses. In this invention, we demonstrate the functional relevance of a novel ligand for CTLA4, the polypeptide designated B7-2, in the initiation of T cell activation.

The present invention provides DNA segments, purified polypeptides, methods for obtaining antibodies, methods of cloning and using recombinant host cells necessary to obtain and use recombinant polypeptides that binds to CTLA4Ig. Accordingly, the present invention concerns generally compositions and methods for the preparation and use of polypeptides other than B7 which binds to CTLA4Ig.

II. Polynucleotides.

A. Polynucleotides that encode a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

In one aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. In a

preferred embodiment, the polynucleotide of the present invention is a DNA molecule. More preferably, the polynucleotide of the present invention encodes a polypeptide designated B7-2.

As used herein, the term "polynucleotide" means a sequence of nucleotides connected by phosphodiester linkages. Polynucleotides are presented herein in the direction from the 5' to the 3' direction. A polynucleotide of the present invention can comprise from about 200 to about several hundred thousand base pairs. Preferably, a polynucleotide comprises from about 300 to about 150,000 base pairs. Preferred lengths of particular polynucleotide are set forth hereinafter.

A polynucleotide of the present invention can be a deoxyribonucleic acid (DNA) molecule or ribonucleic acid (RNA) molecule. Where a polynucleotide is a DNA molecule, that molecule can be a gene or a cDNA molecule. Nucleotide bases are indicated herein by a single letter code: adenine (A), guanine (G), thymine (T), cytosine (C), inosine (I) and uracil (U).

A polynucleotide of the present invention can be prepared using standard techniques well known to one of skill in the art. Exemplary techniques include construction of a library of DNA clones and subsequently screening the library to select a clone that hybridizes to the screening probe. An alternative approach is to express the polypeptide of the present invention in cells such as COS cells transfected with a vector containing the DNA of interest; physical selection of expressing cells by adhesion to antibody-coated dishes, and subsequent cloning of those cells efficiently expressing the polypeptide of the present invention.

Consequently, in one aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation where the polynucleotide is preparable by a process comprising the steps of constructing a library of DNA clones from a cell that expresses the polypeptide; screening the library with a labelled nucleotide probe; and selecting a clone that

hybridizes to the probe. Preferably, the polynucleotide of the invention is prepared by the above process. More preferably, the polynucleotide of the invention encodes a polypeptide that has the amino acid residue sequence of B7-2.

The first step in a cloning procedure is construction of a DNA library. The DNA library can be a genomic library or a cDNA library. Preferably, the library is a cDNA library. Briefly, a cDNA library is constructed by first isolating the total mRNA from a biological sample. Using reverse transcriptase or some other means of amplifying nucleic acids, the mRNA is reverse transcribed to DNA. Short oligonucleotides consisting of restriction enzyme sites, for example, Eco R1, are ligated to the ends of the DNA fragments. These DNA fragments are then ligated into an appropriate vector, for example, pBluescript. The library can then be amplified by transforming an appropriate host cell with the collection of vectors containing difeerent cDNA moleucles. Appropriate hosts are well known in the art and include both eukaryotes and prokaryote, including E. Coli and Yeast. The collection of vectors containing different cDNA molecules is the cDNA library.

The library is then screened for the gene of interest. A common method of screening a library involves the use of an oligonucleotide probe. An oligonucleotide probe is chosen such that the probe can hybridize to the gene of interest. Thus for the present invention an exemplary probe is an oligonucleotide or a fragment thereof that encodes for a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. The probe is labelled with an indicator. Exemplary indicators include radionucleides such as ^{32}P or ^{35}S, fluorescent indicators, and biotin. The labelled probe is hybridized to the library under conditions which promote specific binding. Conditions for hybridizing probe to a library are well known in the art and are described herein. The nucleotide molecule which hybridizes to the probe is then isolated and purified according to steps well known in the art.

An alternative approach to obtain a polynucleotide of the present invention is based on transient expression of the polypeptide of the present invention in COS cells and physical selection of expressing cells by adhesion to antibody-coated dishes. This approach allows a large number of surface antigen cDNAs to be cloned in a short period of time. A major convenience of the method is the recovery of the cDNA of interest in a form containing the necessary sequences for surface expression. Multiple rounds of screening to identify full-length or overlapping clones are thus obviated, and large quantities of antibodies are not required. The use of a high-efficiency cDNA expression vector allows the polypeptide of interest to accumulate at high levels on the surface of transfected cells, thus circumventing the possibility that transfected genomic sequences might fail to generate sufficient levels of polypeptide to allow efficient selection.

Thus, in another embodiment, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation where the polynucleotide is preparable by a process comprising the steps of providing a library of cDNA from cells expressing the polypeptide; transfecting cells with vectors comprising polynucleotides of the library; panning the transfected cells by exposing the cells to an antibody to the polypeptide; identifying those cells expressing the polypeptide, and thus identifying the cDNA sequence comprising the polynucleotide that encodes the polypeptide of the present invention. Preferably, the polynucleotide of the invention is prepared by the above process. More preferably, the polynucleotide of the invention encodes a polypeptide that has the amino acid residue sequence of B7-2.

As an example, the following is an exemplary protocol for preparation of the polynucleotides of the present invention through expression cloning and antibody panning:

(a) Preparation of Adaptors

Resuspend crude adaptors of a concentration of 1 $\mu g/\mu l$, add $MgSO_4$ to 10mM, and precipitate by adding 5 volumes of EtOH. Rinse with 70% ETOH and resuspend in TE at a concentration of $l\mu g/\mu l$. To kinase, take $25\mu l$ of resuspended adaptors, add 3 μl of 10X kinasing buffer and 20 units of kinase: incubate 37° overnight.

(b) Buffers

Loading Buffer:	.5 M LiCl, 10 mM Tris pH 7.5, 1 mM EDTA .1% SDS.
Middle Wash Buffer:	.15 M LiCl, 10 mM Tris PH 7.5, 1 mM EDTA .1% SDS.
RT1 Buffer:	.25 M Tris pH 8.8 (8.2 at 42°), .25 M KC1, 30 mM MgCl2.
RT2 Buffer:	.1 M Tris pH 7.5, 25 mM MgCl2, .5 M KCl, .25 mg/ml BSA, 50 mM DTT.
10X Low Salt:	60 mM Tris pH 7.5, 60 mM MgCl2, 50 mM NaCl, 2.5 mg/ml BSA, 70 mM ME.
10X Ligation Additions:	1 mM ATP, 20 mM DTT, 1 mg/ml BSA 10 MM spermidine.
10X Kinasing Buffer:	.5 M Tris pH 7.5, 10 mM ATP, 20 mM DTT, 10 mM spermidine.
1 mg/ml BSA 100 mM MgCl2.	

(c) Spheroplast Fusion

A set of six fusions requires 100ml of cells in broth. Grow cells containing amplifiable plasmid to OD600=0.5 in LB. Add spectinomycin to 100 $\mu g/ml$ (or chloramphenicol to 150 $\mu g/ml$). Continue incubation at 37° with shaking for 10-16 hours. (Cells begin to lyse with prolonged incubation in spectinomycin or

chloramphenicol medium). Spin down 100ml of culture (JA14/GSA rotor, 250ml bottle) 5 min at 10,000 rpm. Drain well, resuspend pellet in bottle with 5 ml cold 20% sucrose, 50mM Tris-HCl pH 8.0. Add 1ml of 5 mg/ml lysozyme freshly dissolved in 0.25M Tris pH 8. Incubate on ice for 5 min. Add 2 ml cold 0.25M EDTA pH 8.0, incubate 5 min on ice. Add 2ml 50mM Tris pH 8, incubate 5 min at 37° (waterbath). Place on ice, check percent conversion to sphereoplasts by microscopy. In flow hood, slowly add 20 ml of cold DME/10% sucrose/10mM $MgCl_2$ (dropwise, about 2 drops per second). Remove media from cells plated the day before in 6cm dishes (50% confluent). Add 5ml of spheroplast suspension to each dish. Place dishes on top of tube carriers in swinging bucket centrifuge. Up to 6 dishes can be easily prepared at once. (Dishes can be stacked on top of each other, but 3 in a stack is not advisable as the spheroplast layer on top dish is often torn or detached after centrifugation). Spin at 1000 X g (setting 5.7) for 10 min (force is calculated on the basis of the radius to the bottom plate). Aspirate fluid from dishes carefully. Pipet 1.5-2 ml 50% (w/w) PEG1450 (or PEG1000)/50% DME (no serum) into the center of the dish. If necessary, sweep the pipet tip around to insure that the PEG spreads evenly and radially across the whole dish. After PEG has been added to the last dish, prop all of the dishes up (on their lids) so that the PEG solution collects at the bottom. Aspirate the PEG. (The thin layer of PEG that remains on the cells is sufficient to promote fusion; the layer remaining is easier to wash off and better cell viability can be obtained, than if the bulk of the PEG is left behind.) After 90 to 120 seconds (PEG1000) or 120 to 150 seconds (PEG1450) of contact with the PEG solution, pipet 1.5 ml of DME (no serum) into the center of the dish. The PEG layer will be swept radially by the DME. Tilt the dishes and aspirate. Repeat the DME wash. Add 3ml of DME/10% serum containing 15μg/ml gentamicin sulfate. Incubate 4-6 hours in incubator. Remove media and remaining bacterial suspension, add more media and incubate 2-3-days.

Wash briefly and allow the layer to come off in the complete medium at 37 degrees. Although most protocols call for extensive washing of the cell layer to remove PEG, in my experience this washing tends to remove many of the cells

without any substantial benefit. If the cells are allowed to sit in the second DME wash for a few minutes, most of the spheroplast layer will come up spontaneously.

Any commercial source of PEG can be used. Preferably Baker PEG and Kodak PEG is used. (In our experience some lots of Sigma PEG contain significant amounts of aldehyde which intereferes with this protocol.)

The PEG solution can be conveniently prepared by melting a fresh bottle of PEG at 60 degrees and pouring approximate 50 ml aliquots by means of 50 ml centrifuge tube into preweighed bottles (weights marked on bottles). The aliquoted PEG is stored at 5 degrees in the dark. To make up a fresh bottle, weigh the aliquot, remelt, and add an equal volume of DME (no serum). Adjust the pH with 7.5% Na Bicarbonate solution if necessary, and filter sterilize. We have kept the resulting PEG solution up to 3 months at room temperature without detectable adverse consequence.

The PEG can also be melted by microwaving, but this is dangerous, as it is easy to heat the PEG to well over 100 degrees. Superheated bottles of PEG can explode, particularly if the vessel holding PEG is weakened through repeated use or the vessel is not suitable for microwaving solutions.

(d) DEAE Dextran transfection

We typically split the cells to be transfected the night before to give 25-50% confluence. One simple way to achieve this is to resuspend the trypsinized cells from a confluent 10cm dish in 10 ml of medium, and distribute 6 drops from a 10 ml pipet into each 6cm dish to be transfected. The higher the degree of confluence, the greater the ability of the cells to withstand the transfection conditions; however, expression following transfection at high density is typically inferior to that at lower density.

Cells are transfected in DME or IMDM containing 10% heat-inactivated NuSerum (Collaborative Research). NuSerum allows the cells to withstand the transfection condition for greater lengths of time, resulting in improved net expression. If Calf of Fetal Bovine Serum is used, a thick precipitate forms in a substantial fraction of transfections, which results in severely eroded cell viability and expression. Presumably the lower protein concentration in the NuSerum obviates this.

If the cells were split into DME or IMDM/NuSerum, the medium need not be removed for the transfection. Usually 1/5 of a miniprep from 1.5ml of culture is used per 6cm dish in a volume of 1.5-2ml of medium. Chloroquine phosphate and DEAE Dextran are added to 100 micromolar and 400 microgram/ml final concentrations respectively.

After 4 hours at 37 degrees the DEAE-containing medium is removed by aspiration, and 2 ml of 10% DMSO in PBS added. After two minutes or longer at room temperature (timing is not important), the PBS/DMSO is removed, fresh medium is added, and the cells left to incubate for however long is desired before assay (usually two to three days).

It is generally a good idea to check the cells after about 3 hours of exposure to the DEA transfection mix, as their health can decline precipitously. This is particularly true of chloroquine transfections, and it is usually better to shorten the transfection.

Transfection with CsCl-purified DNA can be carried out in the same way (for example for the first round of library screening) but less DNA is required. 20 ng/ml appears to be adequate and higher amounts do not hurt.

(e) Panning

1. Antibody-coated dishes: we use bacteriological 60mm plates, Falcon 1007 or equivalent, or 10cm dishes such as Fisher 8-757-12; we use Sheep anti-mouse affinity purified antibody from Cooper Biomedical (Cappell) and dilute it to 10 micrograms per ml in 50mM Tris HCL, pH 9.5. Add 3ml per 6cm dish, or 10ml per 10cm dish. Let sit about 1.5 hrs, remove to next dish 1.5 hrs. then to 3rd dish. Wash plates 3x with 0.15M NaCl (a wash bottle is convenient for this), incubate with 3ml 1 mg/ml BSA in PBS overnight, aspirate and freeze. (Use freshly coated plates.)

2. Panning Cells are performed in 60mm dishes. Aspirate medium from dish, add 2ml PBS/0.5mM EDTA/0.02% azide and incubate dishes at 37 for 30 min to detach cells from dish. Triturate cells vigorously with short pasteur pipet, and collect cells from each dish in a centrifuge tube. Spin 4 min. setting 2.5 (200 x g). Resuspend cells in .5-1.0 ml PBS/EDTA/azide/5% FBS and add antibodies. Block first for 30 mins with 30 μg αB7 (MG708) then add 5 μg hCTLA4 >30 mins on ice. Incubate >30 min on ice. Add an equal volume of PBS/EDTA/azide, layer on carefully 3 ml PBS/EDTA/Azide/2% Ficoll (7 ml for 10 cm plates). Spin 4 min at setting 2.5. Aspirate supernatant. Take up cell pellet in 0.5 ml PBS/EDTA/azide and add aliquots to antibody coated dishes containing 3ml PBS/EDTA/azide/5% FBS by pipetting through 100 micron Nylon mesh (Tetko). Add cells from at most two 60 mm dishes to one 60 mm antibody coated plate. Let sit at room temperature 1-3 hours. Remove cells that do not adhere to dish by gentle washing with PBS/5% Serum or with medium. Two to three washes of 3 ml are usually sufficient.

3. Hirt Supernatant: Add 0.4 ml 0.6% SDS, 10mM EDTA to panned plate. Let sit 20 minutes (can be as little as 1 min. if there are practically no cells on the plate). Pipet viscous mixture into microfuge tube. Add 0.1 ml 5M NaCl, mix, put on ice at least 5 hrs. Keeping the mixture as cold as possible improves the quality of the Hirt. Spin 4 min, remove supernatant carefully, phenol extract

(twice if the first interface is not clean), add 10 micrograms linear polyacrylamide (or other carrier), fill tube to top with EtOH, precipitate, and resuspend in 0.1ml. Add 3 vol. EtOH/NaOAc, reprecipitate and resuspend in 0.1ml. Transform into MC1061/p3. If the DNA volume exceeds 2% of the competent cell aliquot, the transformation efficiency will suffer. 5% gives the same number of colonies as 2.5% (efficiency is halved.)

Phenol Extract 1x, Phenol/Chloroform Extract lx. Add 10 μg polyacrylamide. ETOH ppt at -70°C for 20-30 mins.
Resuspend in 0.1 ml total volume.
Reprecipitate with 1/10 volume of 3M Sodium acetate and 2½ volume of Ethanol. Resuspend in 0.1 ml water or low salt buffer (e.g TE) and dialyze against water or low salt buffer.

(f) High Efficiency Transformation in *E. coli*

Streak out the desired strain on an LB plate. The next day, inoculate a single colony into 20ml TYM media in a 250ml flask. Grow the cells to midlog phase (OD_{600} ≈0.2-0.8), pour into a 2 liter flask containing 100 ml TYM, continue vigorous agitation until cells grow to 0.5-0.9 OD, then dilute again to 500ml in the same vessel. When cells grow to OD_{600} 0.6, put flask in ice-water, and shake gently to assure rapid cooling. When culture is cool, spin 4.2k rpm. 15 minutes (J6). Pour off supernatant, resuspend pellet in ≈ 100ml cold TfB I media by gentle shaking on ice. Respin in same bottle 4.2krpm, 8 minutes (J6). Pour off supernatant, resuspend pellet in 20ml cold TfB II media by gentle shaking on ice. Aliquot 0.1 to 0.5 ml aliquots in prechilled microfuge tubes, freeze in liquid nitrogen, and store at -70°. For transformation, remove an aliquot, thaw at room temperature until just melting, place on ice, add DNA, let sit on ice 15-30 minutes, incubate at 37° for 5 minutes (6 minutes for 0.5ml aliquots), dilute 1:10 in LB and grow for 90 minutes before plating or applying antibiotic selection. Alternatively the heat-pulsed transformation mix can be plated directly on antibiotic plates onto which a thin (4-5ml) layer of antibiotic-free LB agar has

been poured just before plating. Media and Buffers: TYM: 2% Bacto-Tryptone, 0.5% Yeast Extract, 0.1M NaCl, 10mM $MgSO_4$ (can be added before autoclaving). TfB I: 30mM K0Ac, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15% (v/v) glycerol. TfB II: 10mM Na-MOPS, pH 7.0, 75 mM $CaCl_2$, 10mM KCl, 15% glycerol.

(g) Electroporation

1) Grow 10 ml O/N culture in LB + 10mM $MgCl_2$ + 20μg/ml Kanamycin
2) 2 ml O/N culture into 300 ml LB + 10mM $Mgcl^2$ + 240μl 25 mg/ml kanamycin
3) Grow to $O.D._{600}$ =0.5 (then on ice if necessary), keep cells on ice at all times
4) Spin 2d at 40^c at 2.5 krpm in GSA fixed angle rotor
5) Resuspend in 250ml ice cold H_2O
6) Spin 4Krpm 20' at 40^c in GSA fixed angle rotor, resuspend in 125 ml ice cold H_2O
7) Spin 4Krpm 20' at 40^c in GSA fixed angle rotor
8) Resuspend in 5ml 10% glycerol in ice cold H_2O
9) Spin 3Krpm 10' at 40^c in table top Sorvall
10) Resuspend in 0.5ml 10% glycerol/ice H_2O
11) Use immediately or quick freeze in liq. N_2
12) 40μl cells + 1μl DNA (dialyzed), add DNA immediately before electroporating
13) Pre-cooled cuvett with 1ml SOC media ready in pipetter
14) Pulse controller, 2.5 k volts: 25μFd capacitance: 400 OHMS resistance
15) Press red buttons simultaneously until machine beeps, optimal time constant is 9.3
16) Make sure cells are touching both sides of cuvette.

B. Probes and Primers.

In another aspect, DNA sequence information provided by the present invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of the selected polynucleotide disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of a selected nucleotide sequence of a polypeptide of the present invention. The ability of such nucleic acid probes to specifically hybridize to a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell response lends them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined segment of a gene or polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation from mammalian cells using PCR technology.

To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe molecules that are identical or complementary to at least 10 to 70 contiguous bases of a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality

and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 25 to 40 nucleotides, 55 to 70 nucleotides, or even longer where desired. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, herein incorporated by reference, or by excising selected DNA fragments from recombinant plasmids containing appropriate inserts and suitable restriction enzyme sites.

In another aspect, the present invention contemplates an isolated and purified polynucleotide comprising a base sequence that is identical or complementary to a segment of at least 10 contiguous bases of B7-2, wherein the polynucleotide hybridizes to a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, the isolated and purified polynucleotide comprises a base sequence that is identical or complementary to a segment of at least 25 to 70 contiguous bases of a polynucleotide that encodes for B7-2.

Accordingly, a polynucleotide probe molecule of the invention can be used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids. For example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02 M-0.15 M NaCl at temperatures of 50°C to 70°C. Those conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate a polypeptide, other than B7, that binds to

CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation polypeptide coding sequence from other cells, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. In these circumstances, one can desire to employ conditions such as 0.15 M-0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a polynucleotide of the present invention in combination with an appropriate label for detecting hybrid formation. A wide variety of appropriate labels are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal.

In general, it is envisioned that a hybridization probe described herein is useful both as a reagent in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend as is well known in the art on the particular circumstances and criteria required (e.g., on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe). Following washing of the matrix to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

II. A polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

In one embodiment, the present invention contemplates an isolated and purified polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Preferably, a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation of the invention is a recombinant polypeptide. More preferably, a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation of the present invention is B7-2.

B7-2 is expressed on splenic dendritic cells and is rapidly upregulated on activated B cells. Interestingly, the events controlling the regulation of B7 and B7-2 are distinct. While LPS stimulated B cells expressed B7 only after 48 hours, B7-2 expression could be detected as early as 6 hours. Also, Con A stimulation of splenic B cells resulted in the upregulation of B7-2, but not B7. (See Example 3) This novel ligand is required in the initiation of T cell activation in response to "natural APCs," such as dendritic cells and whole spleen cells.

Extensive PCR analysis of B cells activated for 24 or 48 hours with LPS or Con A indicates that little, if any, B7 is expressed at either time point by the Con A activated cells, while the LPS blasts express B7 mRNA at 48 hours. In addition, preliminary attempts to precipitate B7-2 using CTLA4Ig have been unsuccessful, even though this reagent is quite effective at precipitating B7 from tumor cells and B7 transfectants. This new CTLA4Ig binding molecule plays a critical role in initiating at least T cell activation, and possibly in controlling other T cell responses. The characterization of a novel ligand for CTLA4 is discussed in Example 2.

Even though the expression of both B7 and B7-2 is restricted to professional APCs, the events that control their upregulation are distinct.

Stimulation of the B cells with LPS upregulated both B7 and B7-2, indicating that the same signals may be controlling their expression. However, when Con A was used to stimulate the B cells, only B7-2 was upregulated, demonstrating the existence of distinct signalling pathways. T cell activation via Con A may play a role in either the induction or maintenance of B7-2 expression. Example 3 demonstrates that Con A activation induced higher levels of B7-2 expression on whole spleen cells than on T-depleted spleen cells, while direct activation of B cells with LPS resulted in similar levels of expression on both populations. This may be due to lymphokines released by stimulated T cells which assist in B7-2 induction or may play an important role in maintaining expression. For example, B7 expression via CD40 ligand cross-linking was shown to be enhanced with IL-4. Alternatively, T cell activation may alter a receptor, such as LFA-1, to increase adhesion or alter signalling between the T and B cells, resulting in B7-2's upregulation. However, Con A can also act directly on the B cells to preferentially upregulate B7-2 and not B7. Con A does not induce B cell proliferation. The number of B cells remaining in the Con A stimulated cultures decreased over time. (Example cite?) Yet, Con A must be able to signal the B cell through a cell surface receptor. The CD40/gp39 pathway has been recently shown to induce B7 expression on human B cell lines. (ref) This same pathway may also be able to induce the upregulation of this new ligand or else may represent a distinct pathway used by the B cells to control B7 expression. Con A does not act by this mechanism, since B7 is not upregulated in Con A-stimulated cells. Similarly, Con A does not act through class II, since cross-linking of class II upregulates B7 expression. The induction of B7-2 may occur via the immunoglobulin receptor or other surface receptors.

These results emphasize the complexities involved in T cell activation, and raise the question as to how all these receptor-ligand pairs interact to control immune responses. Recent studies have shown that both CD28 and CTLA4 bind to B7, and that CTLA4 has a 10-20 fold higher affinity for B7 than does CD28. Therefore, B7-2 is also likely to interact with both T cell ligands, and may in fact, have a higher affinity for CD28. Example 4 teaches that both anti-B7 mAB and

hCTLA4Ig inhibit the proliferation of a TH1 clone which expresses B7. We propose one possible model to predict how these ligands all interact with each other under physiological conditions. Upon B cell activation, B7-2 is rapidly upregulated and forms a relevant functional interaction with CD28, which is constitutively expressed on naive T cells. This ligand interaction provides the necessary co-stimulatory signals for T cell activation, resulting in the upregulation of CTLA4 of the T cell 2-3 days later. Concurrently, APCs are upregulating B7 levels, via signalling through MHC class II and CD40. Since CTLA4 has a higher affinity for B7 than does CD28, B7 will preferentially interact with CTLA4 on the activated T cell. This pathway may then send either positive signals to the T cell to maintain an ongoing response, or alternatively may send negative signals to the T cell to shut it down. In fact, we have shown that while hCTLA4Ig has profound inhibitory effects in both transplant and autoimmune models, anti-B7 antibody therapy has either no effect on immune responses in vivo, or in the autoimmune model, exacerbates the disease process. These results indicate that B7 may deliver a negative signal, and that by blocking this signal, an ongoing immune response cannot be downregulated. It is known that costimulatory signals need to be delivered to the T cell within hours of TCR engagement in order to prevent anergy induction. Yet, B7 is not expressed on B cells and macrophages until days after their activation. Our data indicate that the upregulation of B7-2 occurs within six hours of activation, which would allow it to provide the necessary signals to prevent anergy induction. These kinetics and its functional importance in initiating an immune response suggest that B7-2 may be playing an important role in not only stimulating T cells, but may also be acting as an important co-stimulatory molecule responsible for the prevention of anergy induction. Example 5 teaches that T cell activation in the allogeneic MLR depends on B7-2 ligation.

In accordance with standard nomenclature, amino acid residue sequences are denominated by either a single letter or a three letter code as indicated below.

Amino Acid Residue	3-Letter Code	1-Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Modifications and changes can be made in the structure of a polypeptide of the present invention and still obtain a molecule having characteristics a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. For example, certain amino acids can be substituted for other amino acids in a sequence without appreciable loss of regulatory activity. Because it is the interactive capacity and nature of a polypeptide that defines that polypeptide's biological functional activity, certain amino acid sequence substitutions can be made in a polypeptide sequence (or, of

course, its underlying DNA coding sequence) and nevertheless obtain a polypeptide with like properties.

In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art (Kyte & Doolittle, *J. Mol. Biol.*, 157:105-132, 1982). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still result in a polypeptide with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, substrates, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydropathic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly where the biological functional equivalent polypeptide or peptide thereby created is intended for use in immunological embodiments. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a polypeptide, as governed by the hydrophilicity of

its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the polypeptide.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5 ± 1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine (See Table 1, below). The present invention thus contemplates functional or biological equivalents of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation as set forth above.

TABLE 1

Original Residue	Exemplary Substitutions
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg
Met	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Biological or functional equivalents of a polypeptide can also be prepared using site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of second generation polypeptides, or biologically functional equivalent polypeptides or peptides, derived from the sequences thereof, through specific mutagenesis of the underlying DNA. As noted above, such changes can be desirable where amino acid substitutions are desirable. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows

the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by *Adelman, et al. (1983)*. As will be appreciated, the technique typically employs a phage vector which can exist in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (*Messing, et al. 1981*). These phage are commercially available and their use is generally known to those of skill in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes all or a portion of a polypeptide sequence selected. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of *Crea, et al. (1978)*. This primer is then annealed to the singled-stranded vector, and extended by the use of enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as *E. coli* cells and clones are selected which include recombinant vectors bearing the mutation. Commercially available kits come with all the reagents necessary, except the oligonucleotide primers.

A polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation of the present

invention is not limited to a particular source. As disclosed herein, the techniques and compositions of the present invention provide, for example, the identification and isolation of such peptides from mouse sources. Thus, the invention provides for the general detection and isolation of a family of polypeptides from a variety of animal sources where the polypeptide binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

A polypeptide of the present invention is prepared by standard techniques well known to those skilled in the art. Such techniques include, but are not limited to, isolation and purification from tissues known to contain that polypeptide, and expression from cloned DNA that encodes such a polypeptide using transformed cells.

Soluble recombinant B7-2 without an Ig tail can be purified by standard molecule sieve and ion exchange chromotography or using monoclonal antibodies directed against the B7-2 molecule. Similar types of approaches are used to purify the natural B7-2 from cells as well.

Polypeptides that regulate T cell activation are found in virtually all mammals including human. Although it is likely that there exist minor variation in the sequences of such polypeptides in different species where such a differences exist, identification of those differences is well within the skill of an artisan in light of the present invention. Thus, the present invention contemplates a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation from any mammal. A preferred mammal is a rodent or a human.

III. Expression Vectors

In an alternate embodiment, the present invention provides expression vectors comprising polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T

cell activation. Preferably, an expression vector of the present invention comprise a polynucleotide that encode a polypeptide comprising the amino acid residue sequence of B7-2. More preferably, the expression vectors of the invention comprise polynucleotide operatively linked to an enhancer-promoter. Even more preferably, the expression vectors of the invention comprise polynucleotide operatively linked to a prokaryotic promoter. Alternatively, the expression vectors of the present invention comprise polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter, and the expression vectors further comprise a polyadenylation signal that is positioned 3′ of the carboxy-terminal amino acid and within a transcriptional unit of the encoded polypeptide.

A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region or a promoter of a generalized eukaryotic RNA Polymerase transcription unit.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides specificity of time, location and expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding

sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription is controlled, is dependent *inter alia* upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized.

A coding sequence of an expression vector is operatively linked to a transcription terminating region. RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (RNA). Transcription-terminating regions are well known in the art. A preferred transcription-terminating region used in an adenovirus vector construct of the present invention comprises a polyadenylation signal of SV40 or the protamine gene.

An expression vector comprises a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Such a polypeptide is meant to include a sequence of nucleotide bases encoding a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and

regulates T cell activation sufficient in length to distinguish said segment from a polynucleotide segment encoding a polypeptide that does not regulate T cell activation. A polypeptide of the invention can also encode biologically functional polypeptides or peptides which have variant amino acid sequences, such as with changes selected based on considerations such as the relative hydropathic score of the amino acids being exchanged. These variant sequences are those isolated from natural sources or induced in the sequences disclosed herein using a mutagenic procedure such as site-directed mutagenesis.

Preferably, the expression vectors of the present invention comprise polynucleotide that encodes a polypeptide comprising the amino acid residue sequence of B7-2. An expression vector can include a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation coding region itself or it can contain coding regions bearing selected alterations or modifications in the basic coding region of such a polypeptide. Alternatively, such vectors or fragments can code larger polypeptides or polypeptides which nevertheless include the basic coding region. In any event, it should be appreciated that due to codon redundancy as well as biological functional equivalence, this aspect of the invention is not limited to the particular DNA molecules corresponding to the amino acid sequence of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

Exemplary vectors include the mammalian expression vectors of the pCMV family including pCMV6b and pCMV6c (Chiron Corp., Emeryville CA). In certain cases, and specifically in the case of these individual mammalian expression vectors, the resulting constructs can require co-transfection with a vector containing a selectable marker such as pSV2neo. Via co-transfection into a dihydrofolate reductase-deficient Chinese hamster ovary cell line, such as DG44, clones expressing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation can be detected.

A DNA molecule of the present invention can be incorporated into a vector, a number of techniques which are well known in the art. For instance, the vector pUC18 has been demonstrated to be of particular value. Likewise, the related vectors M13mp18 and M13mp19 can be used in certain embodiments of the invention, in particular, in performing dideoxy sequencing.

An expression vector of the present invention is useful both as a means for preparing quantities of the DNA itself, and as a means for preparing the encoded polypeptide and peptides. It is contemplated that where a polypeptide of the invention are made by recombinant means, one can employ either prokaryotic or eukaryotic expression vectors as shuttle systems. However, in that prokaryotic systems are usually incapable of correctly processing precursor polypeptides and, in particular, such systems are incapable of correctly processing membrane associated eukaryotic polypeptides, and since eukaryotic polypeptides are anticipated using the teaching of the disclosed invention, a eukaryotic cell is a preferred host. However, even where the DNA segment encodes a eukaryotic polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, it is contemplated that prokaryotic expression can have some additional applicability. Therefore, the invention can be used in combination with vectors which can shuttle between the eukaryotic and prokaryotic cells. Such a system is described herein which allows the use of bacterial host cells as well as eukaryotic host cells.

Where expression of recombinant polypeptides of the present invention is desired and a eukaryotic host is contemplated, it is most desirable to employ a vector such as a plasmid, that incorporates a eukaryotic origin of replication. Additionally, for the purposes of expression in eukaryotic systems, one desires to position the polynucleotide sequence adjacent to and under the control of an effective eukaryotic promoter such as promoters used in combination with Chinese hamster ovary cells. To bring a coding sequence under control of a promoter, whether it is eukaryotic or prokaryotic, what is generally needed is to position the 5' end of the translation initiation side of the proper translational reading frame of

the polypeptide between about 1 and about 50 nucleotides 3' of or downstream with respect to the promoter chosen. Furthermore, where eukaryotic expression is anticipated, one would typically incorporate an appropriate polyadenylation site into the transcriptional unit which includes the polypeptide.

The pCMV plasmids are a series of mammalian expression vectors of particular utility in the present invention. The vectors are designed for use in essentially all cultured cells and work extremely well in SV40-transformed simian COS cell lines. The pCMV1, 2, 3, and 5 vectors differ from each other in certain unique restriction sites in the polylinker region of each plasmid. The pCMV4 vector differs from these 4 plasmids in containing a translation enhancer in the sequence prior to the polylinker. While they are not directly derived from the pCMV1-5 series of vectors, the functionally similar pCMV6b and c vectors are available from the Chiron Corp. of Emeryville, CA and are identical except for the orientation of the polylinker region which is reversed in one relative to the other.

The universal components of the pCMV plasmids are as follows. The vector backbone is pTZ18R (Pharmacia), and contains a bacteriophage f1 origin of replication for production of single stranded DNA and an ampicillin-resistance gene. The CMV region consists of nucleotides -760 to +3 of the powerful promoter-regulatory region of the human cytomegalovirus (Towne stain) major immediate early gene (*Thomsen et al., 1984; Boshart et al., 1985*). The human growth hormone fragment (hGH) contains transcription termination and poly-adenylation signals representing sequences 1533 to 2157 of this gene (*Seeburg, 1982*). There is an *Alu* middle repetitive DNA sequence in this fragment. Finally, the SV40 origin of replication and early region promoter-enhancer derived from the pcD-X plasmid (*Hin*dII to *Pst*I fragment) described in (*Okayama et al., 1983*). The promoter in this fragment is oriented such that transcription proceeds away from the CMV/hGH expression cassette.

The pCMV plasmids are distinguishable from each other by differences in the polylinker region and by the presence or absence of the translation enhancer. The starting pCMV1 plasmid has been progressively modified to render an increasing number of unique restriction sites in the polylinker region. To create pCMV2, one of two *Eco*RI sites in pCMV1 were destroyed. To create pCMV3, pCMV1 was modified by deleting a short segment from the SV40 region (*Stu*I to *Eco*RI), and in so doing made unique the *Pst*I, *Sal*I, and *Bam*HI sites in the polylinker. To create pCMV4, a synthetic fragment of DNA corresponding to the 5'-untranslated region of a mRNA transcribed from the CMV promoter was added C. The sequence acts as a translational enhancer by decreasing the requirements for initiation factors in polypeptide synthesis (*Jobling et al., 1987); Browning et al., 1988*). To create pCMV5, a segment of DNA (*Hpa*I to *Eco*RI) was deleted from the SV40 origin region of pCMV1 to render unique all sites in the starting polylinker.

The pCMV vectors have been successfully expressed in simian COS cells, mouse L cells, CHO cells, and HeLa cells. In several side by side comparisons they have yielded 5- to 10-fold higher expression levels in COS cells than SV40-based vectors. The pCMV vectors have been used to express the LDL receptor, nuclear factor 1, G_s alpha polypeptide, polypeptide phosphatase, synaptophysin, synapsin, insulin receptor, influenza hemmagglutinin, androgen receptor, sterol 26-hydroxylase, steroid 17- and 21-hydroxylase, cytochrome P-450 oxidoreductase, beta-adrenergic receptor, folate receptor, cholesterol side chain cleavage enzyme, and a host of other cDNAs. It should be noted that the SV40 promoter in these plasmids can be used to express other genes such as dominant selectable markers. Finally, there is an ATG sequence in the polylinker between the *Hind*III and *Pst*I sites in pCMU that can cause spurious translation initiation. This codon should be avoided if possible in expression plasmids. A paper describing the construction and use of the parenteral pCMV1 and pCMV4 vectors has been published (Anderson et al., 1989b).

IV. Transfected Cells.

In yet another embodiment, the present invention provides recombinant host cells transformed or transfected with polynucleotide that encode a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, as well as transgenic cells derived from those transformed or transfected cells. Preferably, the recombinant host cells of the present invention are transfected with polynucleotide of B7-2. Means of transforming or transfecting cells with exogenous polynucleotide such as DNA molecules are well known in the art and include techniques such as calcium-phosphate- or DEAE-dextran-mediated transfection, protoplast fusion, electroporation, liposome mediated transfection, direct microinjection and adenovirus infection (*Sambrook, Fritsch and Maniatis, 1989*).

Preferred host cells include tumor cells in order to induce tumor-specific immune responses, antigen-presenting cells to increase the immunogenicity of foreign antigens for things like vaccines, or non-classical antigen-presenting cells such as epithelial or endothelial cells that could provide good antigen-presenting function.

The most widely used method is transfection mediated by either calcium phosphate or DEAE-dextran. Although the mechanism remains obscure, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transported to the nucleus. Depending on the cell type, up to 90% of a population of cultured cells can be transfected at any one time. Because of its high efficiency, transfection mediated by calcium phosphate or DEAE-dextran is the method of choice for experiments that require transient expression of the foreign DNA in large numbers of cells. Calcium phosphate-mediated transfection is also used to establish cell lines that integrate copies of the foreign DNA, which are usually arranged in head-to-tail tandem arrays into the host cell genome.

In the protoplast fusion method, protoplasts derived from bacteria carrying high numbers of copies of a plasmid of interest are mixed directly with cultured mammalian cells. After fusion of the cell membranes (usually with polyethylene glycol), the contents of the bacteria are delivered into the cytoplasm of the mammalian cells and the plasmid DNA is transported to the nucleus. Protoplast fusion is not as efficient as transfection for many of the cell lines that are commonly used for transient expression assays, but it is useful for cell lines in which endocytosis of DNA occurs inefficiently. Protoplast fusion frequently yields multiple copies of the plasmid DNA tandemly integrated into the host chromosome.

The application of brief, high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

Liposome transfection involves encapsulation of DNA and RNA within liposomes, followed by fusion of the liposomes with the cell membrane. The mechanism of how DNA is delivered into the cell is unclear but transfection efficiencies can be as high as 90%.

Direct microinjection of a DNA molecule into nuclei has the advantage of not exposing DNA to cellular compartments such as low-pH endosomes. Microinjection is therefore used primarily as a method to establish lines of cells that carry integrated copies of the DNA of interest.

This invention further contemplates the use of viral based vectors to deliever genes of interest to a target cell. A viral vector should satisfy the following criteria: 1) the vector should be capable of infecting the target cell; 2) the vector should be non-pathogenic, that is, avirulent and not reactivatible to cause infection; 3) the vector can deliever the gene of interest to the target cell; and 4) the gene of interest is expressed in the target cell. The B7-2 cDNA can be cloned into the viral genome by the use of standard molecular biology techniques.

Viruses can vary greatly in their specificity for a host cell. Vaccinia virus can infect most mammalian cell types. In contrast, Herpes Simplex virus (HSV-1) has a natural tropism for human central nervous tissue. The specific cell types that viruses can infect are well known in the art. Based upon the target cell type, an appropriate viral vector can be chosen. Appropriate viral vectors include but are not limited to adenovirus, picorna virus, vaccinia virus, herpes virus, corona virus, eunyvirus, togavirus, hantavirus, rhabdovirus and retroviruses.

The viral vector should be avirulent. One method of preparing avirulent virus is to manipulate the viral genome to render the virus non-pathogenic. The methods for creating such mutations are set forth in detail in U.S. Patent No. 4,769,331, incorporated herein by reference. As a specific example, it has been reported that the α4 gene in herpes simplex virus (HSV) is required for the virus to replicate. De Luca, et. al *J. Virol.*, **56**, 558-570 (1985). A HSV vector with a mutational lesion in the α4 gene is constructed. The proposed virus will no longer be able to replicate, multiply and reactivate from latent infection in the CNS. The virus can, in the absence of α4 gene, establish a latent infection in the neuron. This virus can be obtained by co-transfection of viral DNA with plasmid containing a α4 expressing cell line. Id. The use of adenovirus as a vector for cell transfection is well known in the art. Adenovirus vector-mediated cell transfection has been reported for various cells (*Stratford-Perricaudet, et al. 1992*).

The viral vector containing a B7-2 cDNA can be combined with a pharmaceutically acceptable carrier such as buffered saline and injected at an appropriate site. As will be recognized by those skilled in the medical arts the amount of virus administered will vary depending upon several factors, including the vector's ability to target the cells requiring treatement, the extent to which the gene is expressed in the target tissue, and the activity of the expressed protein, among others. An innoculum containing approximately 10^3 - 10^6 viruses in phosphate buffered saline or skim milk has produced successful results in mice.

A transfected cell can be prokaryotic or eukaryotic. Preferably, the host cells of the invention are eukaryotic host cells. More preferably, the recombinant host cells of the invention are COS-1 cells. Where it is of interest to produce a human polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, cultured mammalian or human cells are of particular interest.

In another aspect, the recombinant host cells of the present invention are prokaryotic host cells. Preferably, the recombinant host cells of the invention are bacterial cells of the DH5α strain of *Escherichia coli*. In general, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, *E. coli* K12 strains can be particularly useful. Other microbial strains which can be used include *E. coli* B, and *E. coli* X1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes can also be used for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilus*, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratus marcesans*, and various *Pseudomonas* species can be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* can be transformed using pBR322, a plasmid derived from an *E. coli* species (*Bolivar, et al. 1977*). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own polypeptides.

Those promoters most commonly used in recombinant DNA construction include the ß-lactamase (penicillinase) and lactose promoter systems (*Chang, et al. 1978; Itakura, et al. 1977; Goeddel, et al. 1979; Goeddel, et al. 1980*) and a tryptophan (TRP) promoter system (EPO Appl. Publ. No. 0036776; Siebwenlist et al., 1980). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to introduce functional promoters into plasmid vectors (*Siebwenlist, et al. 1980*).

In addition to prokaryotes, eukaryotic microbes, such as yeast can also be used. *Saccharomyces cerevisiase* or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (*Stinchcomb, et al. 1979; Kingsman, et al. 1979; Tschemper, et al. 1980*). This plasmid already contains the *trp*1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (*Jones, 1977*). The presence of the *trp*1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (*Hitzeman, et al. 1980*) or other glycolytic enzymes (*Hess, et al. 1968; Holland, et al. 1978*) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also introduced into the expression vector downstream from the sequences to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin or replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms can also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Kruse and Peterson, 1973*). Examples of such useful host cell lines are AtT-20, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COSM6, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often derived from viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, Cytomegalovirus and most frequently

Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (*Fiers, et al. 1978*). Smaller or larger SV40 fragments can also be used, provided there is included the approximately 250 bp sequence extending from the *Hin*dIII site toward the *Bgl*I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication can be provided with by construction of the vector to include an exogenous origin, such as can be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV, CMV) source, or can be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

V. Preparing a Recombinant polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

In yet another embodiment, the present invention contemplates a process of preparing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising transfecting cells with polynucleotide that encode the said polypeptide to produce transformed host cells; and maintaining the transformed host cells under biological conditions sufficient for expression of the polypeptide. Preferably, the transformed host cells are eukaryotic cells. More preferably still, the eukaryotic cells are COS-1 cells. Alternatively, the host cells are prokaryotic cells. More preferably, the prokaryotic cells are bacterial cells of the DH5α strain of *Escherichia coli*. Even more preferably, the polynucleotide transfected into the transformed cells comprise the nucleotide base sequence of that encodes for B7-2.

Most preferably transfection is accomplished using a hereinbefore disclosed expression vector.

A host cell used in the process is capable of expressing a functional polypeptide of the present invention. A preferred host cell is a Chinese hamster ovary cell. However, a variety of cells are amenable to a process of the invention, for instance, yeasts cells, human cell lines, and other eukaryotic cell lines known well to those of the art.

Following transfection, the cell is maintained under culture conditions for a period of time sufficient for expression of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Culture conditions are well known in the art and include ionic composition and concentration, temperature, pH and the like. Typically, transfected cells are maintained under culture conditions in a culture medium. Suitable medium for various cell types are well known in the art. In a preferred embodiment, temperature is from about 20°C to about 50°C, more preferably from about 30°C to about 40°C and, even more preferably about 37°C.

pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. Osmolality is preferably from about 200 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably from about 290 mosm/L to about 310 mosm/L. Other biological conditions needed for transfection and expression of an encoded protein are well known in the art.

Transfected cells are maintained for a period of time sufficient for expression of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. A suitable time depends *inter alia* upon the cell type used and is readily determinable by a skilled artisan. Typically, maintenance time is from about 2 to about 14 days.

Recombinant polypeptide is recovered or collected either from the transfected cells or the medium in which those cells are cultured. Recovery comprises isolating and purifying the polypeptide of the present invention. Isolation and purification techniques for polypeptides are well known in the art and include such procedures as precipitation, filtration, chromatography, electrophoresis and the like.

VI. Antibodies.

In still another embodiment, the present invention provides antibodies immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Means for preparing and characterizing antibodies are well known in the art (*See, e.g., Antibodies "A Laboratory Manual,* E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide may vary in its immunogenicity. It is often necessary therefore to couple the immunogen (e.g., a polypeptide or polynucleotide) of the present invention) with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogencity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies *inter alia,* upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal. The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

In another aspect, the present invention contemplates a process of producing an antibody immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising the steps of (a) transfecting recombinant host cells with polynucleotide that encodes a polypeptide of the present invention; (b) culturing the host cell under conditions sufficient for expression of the polypeptides; (c) recovering the polypeptides; and (d) preparing the antibodies to the polypeptides. Preferably, the host cell is transfected with the polynucleotide that encodes for B7-2. Even more preferably, the present invention provides antibodies prepared according to the process described above.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as those exemplified in U.S. Pat. No

4,196,265, herein incorporated by reference. Typically, a technique involves first immunizing a suitable animal with a selected antigen (e.g., a polypeptide or polynucleotide of the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media is supplemented with hypoxanthine.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200 μg of an antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*. At some time (e.g., at least two

weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 10^7 to 2 X 10^8 lymphocytes.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus denominated immortal. Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are *hybridoma* cells. Like myeloma cells, hybridoma cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotide of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotide can be isolated and purified by techniques such as *antibody-affinity chromatography*. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

VII. Pharmaceutical Compositions.

In a preferred embodiment, the present invention provides pharmaceutical compositions comprising a polypeptide or a polynucleotide of the present invention and physiologically acceptable carriers. More preferably, the pharmaceutical compositions comprise the polypeptide B7-2 or a polynucleotide that encodes B7-2.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes intravenous, intramuscular, intraarterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminants, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

A carrier can also be a liposome. Means for using liposomes as delivery vehicles are well known in the art [*See, e.g. Gabizon et al., 1990; Ferruti et al., 1986; and Ranade, V.V., 1989*].

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (e.g. injected intravascularly).

VIII. Detecting Polynucleotides and the Polypeptides Encoded.

Alternatively, the present invention provides a process of detecting a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises immunoreacting the polypeptides with antibodies prepared according to the process described above to form antibody-polypeptide conjugates, and detecting the conjugates.

In yet another embodiment, the present invention contemplates a process of detecting messenger RNA transcripts that encode a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises (a) hybridizing the messenger RNA transcripts with polynucleotide sequences that encode a polypeptide of the present invention to form duplexes; and (b) detecting the duplex. Alternatively, the present invention provides a process of detecting DNA molecules that encode a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, wherein the process comprises (a) hybridizing DNA molecules with polynucleotide that encode a polypeptide of the present invention to form duplexes; and (b) detecting the duplexes.

IX. Screening Assays

In yet another aspect, the present invention contemplates a process of screening substances for their ability to bind to a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation comprising the steps of providing a polypeptide of the present invention, and testing the ability of selected substances to interact with a polypeptide of the present invention.

Utilizing the methods and compositions of the present invention, screening assays for the testing of candidate substances which affect T cell activation can be derived. A candidate substance is a substance which potentially can promote or inhibit T cell activation by binding to a polypeptide other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins,.

A screening assay of the present invention involves the determination of the ability of a candidate substance to inhibit or promote T cell activation. Target cells include cells that endogenously produce a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. Alternatively, a target cell can be transformed with a polynucleotide that encodes a polypeptide of the present invention, to produce a cell that expresses a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. It is further contemplated that immortal cell lines can be used as a target cell.

As is well known in the art, a screening assay provides a cell under conditions suitable for testing the ability of a candidate substance to regulate T cell activation. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant co-factors, and relevant modifications to the polypeptide such as glycosylation or prenylation. It is contemplated that a polypeptide of the present invention can be expressed and utilized in a prokaryotic or eukaryotic cell. The host cell can be fractionated into sub-cellular fractions where the polypeptide of the present invention can be found. For example, cells can be fractionated into the nucleil, the endoplasmic reticulum, vesicles, or the membrane surfaces of the cell.

pH is preferably from about a value of 6.0 to a value of about 8.0, more preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. In a preferred embodiment, temperature is from about 20°C to about 50°C, more preferably from about 30°C to about 40°C and, even more preferably about 37°C. Osmolality is preferably from about 5 milliosmols per

liter (mosm/L) to about 400 mosm/l and, more preferably from about 200 milliosmols per liter to about 400 mosm/l and, even more preferably from about 290 mosm/L to about 310 mosm/L.

It is well known in the art that proteins can be reconstituted in artificial membranes, vesicles or liposomes. (*Danboldt, et al. 1990*). The present invention contemplates that a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation can be incorporated into artificial membranes, vesicles or liposomes. The reconstituted polypeptide of the present invention can be utilized in screening assays.

A. Screening assays for a polypeptide of the present invention.

The present invention provides a process of screening a biological sample for the presence of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. A biological sample to be screened can be a biological fluid such as extracellular or intracellular fluid or a cell or tissue extract or homogenate. A biological sample can also be an isolated cell (e.g., in culture) or a collection of cells such as in a tissue sample or histology sample. A tissue sample can be suspended in a liquid medium or fixed onto a solid support such as a microscope slide.

In accordance with a screening assay process, a biological sample is exposed to an antibody immunoreactive with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation whose presence is being assayed. Typically, exposure is accomplished by forming an admixture in a liquid medium that contains both the antibody and the candidate polypeptide. Either the antibody or the sample with the candidate polypeptide can be affixed to a solid support (e.g., a column or a microtiter plate).

The biological sample is exposed to the antibody under biological reaction conditions and for a period of time sufficient for antibody-polypeptide conjugate formation. Biological reaction conditions include ionic composition and concentration, temperature, pH and the like.

Ionic composition and concentration can range from that of distilled water to a 2 molal solution of NaCl. Preferably, osmolality is from about 100 mosmols/l to about 400 mosmols/l and, more preferably from about 200 mosmols/l to about 300 mosmols/l. Temperature preferably is from about 4°C to about 100°C, more preferably from about 15°C to about 50°C and, even more preferably from about 25°C to about 40°C. pH is preferably from about a value of 4.0 to a value of about 9.0, more preferably from about a value of 6.5 to a value of about 8.5 and, even more preferably from about a value of 7.0 to a value of about 7.5. The only limit on biological reaction conditions is that the conditions selected allow for antibody-polypeptide conjugate formation and that the conditions do not adversely affect either the antibody or the polypeptide.

Exposure time will vary *inter alia* with the biological conditions used, the concentration of antibody and polypeptide and the nature of the sample (e.g., fluid or tissue sample). Means for determining exposure time are well known to one of ordinary skill in the art. Typically, where the sample is fluid and the concentration of polypeptide in that sample is about 10^{-10} M, exposure time is from about 10 minutes to about 200 minutes.

The presence of a polypeptide in the sample is detected by detecting the formation and presence of antibody-polypeptide conjugates. Means for detecting such antibody-antigen conjugates or complexes are well known in the art and include such procedures as centrifugation, affinity chromatography and the like, binding of a secondary antibody to the antibody-candidate complex.

In one embodiment, detection is accomplished by detecting an indicator affixed to the antibody. Exemplary and well known such indicators include

radioactive labels (e.g., ^{32}P, ^{125}I, ^{14}C), a second antibody or an enzyme such as horse radish peroxidase. Means for affixing indicators to antibodies are well known in the art. Commercial kits are available.

B. Screening assay for polynucleotide that a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

A DNA molecule and, particularly a probe molecule, can be used for hybridizing as oligonucleotide probes to a DNA source suspected of possessing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation encoding polynucleotide or gene. The probing is usually accomplished by hybridizing the oligonucleotide to a DNA source suspected of possessing such a gene. In some cases, the probes constitute only a single probe, and in others, the probes constitute a collection of probes based on a certain amino acid sequence or sequences of a polypeptide of the present invention and account in their diversity for the redundancy inherent in the genetic code.

A suitable source of DNA for probing in this manner is capable of expressing a polypeptide of the present ivnention and can be a library of a cell line of interest. Alternatively, a source of DNA can include total DNA from the cell line of interest. Once the hybridization process of the invention has identified a candidate DNA segment, one confirms that a positive clone has been obtained by further hybridization, restriction enzyme mapping, sequencing and/or expression and testing.

Alternatively, such DNA molecules can be used in a number of techniques including their use as: (1) diagnostic tools to detect normal and abnormal DNA sequences in DNA derived from patient's cells; (2) means for detecting and isolating other members of the regulatory peptide family and related polypeptides from a DNA library potentially containing such sequences; (3) primers for

hybridizing to related sequences for the purpose of amplifying those sequences; (4) primers for altering the native polypeptide DNA sequences; as well as other techniques which rely on the similarity of the DNA sequences to those of the DNA sequence of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

As set forth above, in certain aspects, DNA sequence information provided by the invention allows for the preparation of relatively short DNA or RNA sequences (e.g., probes) that specifically hybridize to encoding sequences of an T cell regulatory gene. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the encoding sequence of a polypeptide of the present invention, for example, a sequence that encodes for B7-2. The ability of such nucleic acid probes to specifically hybridize to other encoding sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

To provide certain of the advantages in accordance with the invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe sequences that are complementary to at least a 14 to 40 or so long nucleotide stretch of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation. A particularly preferred nucleotide sequence is a sequence that encodes for B7-2. A size of at least 14 nucleotides in length helps to ensure that the fragment is of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 14 bases in length are generally preferred, though, to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 14 to 20 nucleotides, or even longer where desired.

Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, herein incorporated by reference, or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate coding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. Under such circumstances, one employs conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a nucleic acid sequence of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one likely employs an enzyme tag such a urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein are useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the sample containing test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend *inter alia* on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

X. Assay kits.

In another aspect, the present invention contemplates diagnostic assay kits for detecting the presence of a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation in biological samples, where the kits comprise a first container containing a first antibody capable of immunoreacting with the polypeptide, with the first antibody

present in an amount sufficient to perform at least one assay. Preferably, the assay kits of the invention further comprise a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in the assay kits of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

The present invention also contemplates a diagnostic kit for screening agents. Such a kit can contain a polypeptide of the present invention. The kit can contain reagents for detecting an interaction between an agent and a polypeptide of the present invention. The provided reagent can be radiolabelled. The kit can contain a known radiolabelled agent capable of binding or interacting with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

In an alternative aspect, the present invention provides diagnostic assay kits for detecting the presence, in biological samples, of polynucleotide that encode a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases that encodes for B7-2.

In another embodiment, the present invention contemplates diagnostic assay kits for detecting the presence, in a biological sample, of antibodies immunoreactive with a polypeptide of the present invention, the kits comprising a first container containing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation that immunoreacts with the antibodies, with the polypeptides present in an amount sufficient to perform at least one assay. The reagents of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous

solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatograph media or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent. The solvent can be provided.

The following examples have been included to illustrate preferred modes of the invention. Certain aspects of the following examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the invention. These examples are exemplified through the use of standard laboratory practices of the inventor. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following examples are intended to be exemplary only and that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

EXAMPLES

Example 1: *The anti-B7 antibodies have equivalent or higher affinity for B7 than does hCTLA4Ig.*

A. Preparation of Antibodies and Recombinant Proteins.

Human CTLA4Ig which was biotinylated and used as a staining reagent was prepared by techniques well known in the art. Uncoupled human CTLA4Ig used as a blocking reagent in both the staining and proliferation studies was prepared as follows. The coding sequence for the extracellular portion of human CTLA4 was joined to the hinge-CH2-CH3 domains derived from a human genomic IgG1 gene by PCR, using a strategy similar to that previously reported. The hCTLA4Ig genetic fusion was cloned into expression vector pNADSH (Repligen Corp., Cambridge, MA) under control of a modified CMV promoter. The vector also contained the genes for G418 resistance and for methotrexate amplification (DHFR). Chinese hamster ovary cells (CHO, DG44; DHFR-) were transfected by electroporation and the cells cultured in MEM Alpha medium

lacking nucleosides (Gibco, Grand Island, NY) with G418 added to a final active concentration of 500 μg/ml. CHO transfectants expressing CTLA4Ig were identified by ELISAs specific for both the Ig portion of the fusion protein and for the CTLA4 domain. The CTLA4Ig was purified from 10 fold concentrated culture supernatants by binding to immobilized protein A (IPA-300, Repligen Corp., Cambridge, MA) followed by elution with 0.1 M sodium citrate, pH 3 and neutralized immediately with 0.1 M Tris base. Pooled fractions were dialyzed overnight against 0.5 x PBS at 4°C. The CTLA4Ig was analyzed by SDS-PAGE and was detected by Coommassie blue staining and by western blots using anti-CTLA4 antiserum R1438. The purified CTLA4Ig existed primarily as a dimer and was able to immunoprecipitate hB7Ig and to stain CHO cells expressing human B7 on their surface. Control human Ig and 16-10A1 (a hamster anti-murine B7 mAb) were provided by Repligen Corporation (Cambridge, MA). 1G10 (a rat IgG2a anti-murine B7) has been described previously. FITC-coupled anti-B220 mAb (clone RA3-6B2, a rat IgG2a) was purchased from PharMingen (San Diego, CA). FITC-coupled goat anti-hamster was purchased from Jackson Immunological Research (West Grove, PA). PE-AV was obtained from Southern Biotechnology (Birmingham, AL). N418 (clone HB224) is a hamster anti murine-CD11c that recognizes dendritic cells. This mAb was a generous gift from Dr. Frank Fitch (Univ. of Chicago, Chicago, IL). Human CTLA4Ig and 16-10A1 were biotinylated and 1G10 was FITC conjugated as described below. 2.4G2 is a rat anti-mouse FcR mAb which has been previously described.

B. Results

Initial studies were performed to determine the epitope specificity and relative affinites of hCTLA4Ig and of two different anti-B7 mAbs, using a CHO cell line transfected with murine B7 (B7-tf CHO). As shown in figure 1 (panels A,E,I), all three reagents were able to bind to the B7-transfected cells, and this binding was not inhibited by control Ig. Both of the anti-B7 mAbs (1G10 and 16-10A1) inhibited the binding of hCTLA4Ig-biotin to the B7-tf CHO cells with equal or greater effectiveness than hCTLA4Ig (panels A-D). For instance, the

1G10 antibody blocked the binding of hCTLA4Ig-biotin to B7 at similar concentrations as hCTLA4Ig (panels B vs. D), whereas the 16-10A1 mAb completely blocked hCTLA4Ig staining, even at concentrations 100-fold less than the amount of hCTAL4Ig needed for equivalent blocking of CTLA4Ig-biotin binding (panels B vs. C). Similar results were obtained in reciprocal blocking studies (figure 1, panels E-H and I-L). The binding of 16-10A1 was only weakly inhibited by both hCTLA4Ig and 1G10, whereas the binding of 1G10 was inhibited equally by 1G10 and hCTLA4Ig, but significantly better by 16-10A1. These data demonstrate that the anti-B7 mAbs. have equivalent if not greater affinity for B7 than does hCTLA4Ig, and that these reagents bind to overlapping epitopes on the B7 molecule.

Example 2: *A novel ligand for CTLA4 is expressed on activated splenic B cells and dendritic cells.*

A. Flow Cytometric Analysis (FCM).

Purified CTLA4Ig and 16-10A1 were biotinylated as previously described. Purified was 1G10 was FITC-coupled as follows. Fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) was dissolved in N,N-dimethyl formamide (DMF) (Fisher Scientific) to give a 10 mg/ml solution. FITC/DMF was added to purified mAb, 1G10, at 1:10 w/w and incubated at 25°C for four hours, followed by dialysis into PBS containing an anion exchange resin (AG1-X8, 200-400 mesh, chloride form; Bio-Rad). Aggregates were removed prior to use by airfuge centrifugation (Becton-Dickinson).

B. Dendritic Cell Purification.

Single cell suspensions of the various cell lines and spleen were prepared by established methods. 10^5 cells were incubated with 2.4G2 for 15 minutes prior to staining to prevent antibody binding to Fc receptors. The cells were then washed and pre-incubated with uncoupled antibodies where indicated for 30 minutes at 4°C prior to adding fluorochrome-coupled reagents for 45 minutes to 1

hour at the following concentrations: 2.5 μg/ml biotinylated hCTLA4Ig, 1.0 μg/ml biotinylated 16-10A1 (anti-B7), or 4.0 μg/ml FITC-coupled 1G10 (anti-B7). The cells were washed and biotinylated reagents were counterstained with phycroerythin-conjugated streptavidin (PE-SA, Southern Biotechnology (Birmingham, AL). One and two-color FCM were performed using a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA), interfaced to a Hewlett-Packard 310 computer. Data analyses were performed using Consort-30 software (Becton-Dickinson). Logarithmically-amplified fluorescence data were collected on 10,000 viable cells as determined by forward and right angle light scatter intensity.

C. Mice

C57BL/6 and DBA/2J mice (8 to 12 week old male mice) and BALB/cJ mice (6-8 week old female mice) were obtained from Jackson Laboratories, (Bar Harbor, ME) and housed in a specific pathogen free animal barrier facility.

D. Results

The ability of anti-B7 antibodies to inhibit hCTLA4Ig staining of B7 on B7-tf CHO cells was used to screen for the expression of unique ligands for CTLA4 on different splenic populations. We initially analyzed fresh spleen cells and found that no more than 5% of splenocytes stained with either hCTLA4Ig or anti-B7 mAb (Figure 2, panels A-D). The small number of positively staining cells were not T cells, but either a small number of activated B cells or dendritic cells which have been previously shown to express B7 constitutively (ref). In repeated experiments, a small number of B cells reacted preferentially with anti-B7 mAbs, but less well with hCTLA4Ig (panel A vs. B). This binding was blocked by anti-B7 mAbs, but not control Ig. We next examined enriched dendritic cells for expression of either B7 or addition ligands for CTLA4.

Spleens were harvested and depleted of red blood cells with ammonium chloride potassium (ACK) lysing buffer. 10^8 cells were resuspended in 10 mls. of DMEM with 10% FCS, placed in a 100 cm tissue culture dish (Falcon 3003) and incubated at 37°C for 1-1.5 hours. Non-adherent cells were then collected by washing several times with warm DMEM, supplemented with 10% FCS. Following the addition of 10 mls of media, plates were incubated for an additional 2 hours at 37°C and then washed as above. Finally, an additional 10 mls of media was added and the plates were incubated overnight at 37°C.

Both macrophages and dendritic cells adhere to plastic during the initial incubation, but only the dendritic cells detach from the plates after overnight incubation. The dendritic cells collected from the plates were further enriched over a 50%/65% percoll gradient and analyzed for purity by FCM analysis using the dendritic cell-specific mAb, N418.

Dendritic cells can be stained with both hCTLA4Ig and anti-B7 mAb indicating these cells expressed B7 (figure 2, panels I & J). However, hCTLA4Ig staining was not blocked by unlabelled anti-B7 mAb (16-10A1), at a concentration which completely blocked CTLA4Ig binding to the B7-tf CHO cells (figure 1), indicating that an additional ligand for CTLA4Ig was expressed on these cells (panels K and L). Thus, both B7 and a novel CTLA4 ligand are highly expressed on dendritic cells; and therefore, may explain why these cells are such potent stimulators of an immune response.

Since activated B cells have also been shown to upregulate expression of B7, we looked at LPS splenic blasts for the expression of this novel ligand. Stimulation of spleen cells with LPS for three days resulted in the upregulation of B7 on approximately 50% of the splenic B cells (figure 2, panel F). Importantly, greater than 70% of the LPS-activated B cells reacted with the hCTLA4Ig (panel E), indicating this novel ligand was also being upregulated on activated B cells. Once again blocking studies demonstrated that unlabelled anti-B7 mAb (16-10A1) only minimally inhibited CTLA4Ig staining of LPS activated B cells (figure 2,

panels E vs. G and H). The lower affinity anti-B7 mAb, 1G10, also did not inhibit hCTLA4Ig binding to either dendritic cells or the activated B cells. It should be noted that both of the unlabeled anti-B7 mAbs blocked the binding of FITC or biotin coupled anti-mB7 mAbs to the activated B cells and dendritic cells. Thus, these results suggested that LPS-activation of B cells upregulated B7 expression on a subset of B cells but, in addition, a new ligand for CTLA4 was induced on LPS-activated B cells, including a subset of B cells that did not express detectable levels of B7. We refer to this novel ligand as B7-2.

Example 3: *Differential regulation of the B7-2 and B7 expression on activated splenic B cells.*

A. T-Cell Depletion.

Spleen cells, depleted of red blood cells by ACK lysis, were resuspended at 2×10^7 cells/ml in a 1:1 ratio of anti-thy1 mAb supernatent (AT83A) and PBS. This mixture was kept at 4°C for 20 minutes and then rabbit complement (Pel-Freeze) was added at a 1:20 titer. This was incubated in a 37°C water bath for one hour. After complement killing, cells were washed once with DMEM, viable cells were separated by ficoll-hypaque, and washed twice with DMEM before use. The purity of the T-cell depleted population was examined by FCM using anti-TCR-FITC (H597).

B. Results.

B7 is maximally expressed on LPS-activated B cells after 48-72 hours. Since a costimulator molecule which influences primary T cell responses might be expected to be expressed early following B cell engagement, we compared the time course of expression of B7-2 and B7 following LPS activation.

As expected, B7 was induced on splenic B cells stimulated with LPS, with maximal levels of expression observed by day 3. This was shown by staining with both anti-B7 mAbs (Figure 3, panel A. However, CTLA4Ig binding was observed

as early as 6 hours after LPS stimulation of splenic B cells. The CTLA4Ig reactivity was not blocked by inhibitory amounts of unlabelled anti-mB7 mAbs, confirming that CTLA4Ig was binding to B7-2 and not B7. Expression of CTLA4Ig-1 reached maximal levels of expression by 24 hours and remained highly expressed until day 4, when CTLA4Ig binding began to decrease.

We next examined whether B7-2 and B7 were upregulated on T or B cells following activation with Con A, a known potent T cell mitogen. Following Con A activation of spleen cells, CTLA4Ig binding was observed on B cells (figure 3, panel A), but not T cells. Surprisingly, CTLA4Ig binding was observed as early as 6 hours following Con A addition, suggesting that Con A may have directly bridged T and B cells, changed the confirmation of a T or B cell surface ligand resulting in altered T/B cell interactions, or directly acted on the B cell population. To differentiate between these possibilities, spleen cells were T-depleted by anti-Thy1-mAb plus complement to remove >99% of the T cells.

Con A was added to the T-depleted population and CTLA4Ig binding was examined. As seen in figure 3, panel B, Con A induced equivalent amounts of B7-2 after 24 hours in culture, as did LPS, and the time course of expression was similar. Interestingly, Con A activated spleen cells expressed nearly two times more B7-2 than did spleen cells which had been depleted of T cells, whereas LPS stimulated whole or T-depleted spleen expressed similar levels of the ligand (figure 3, panel B). Thus, it is likely that Con A activation of T cells augments B7-2 induction, possibly through lymphokines or cell-cell interactions. Unlike the LPS-induced B cell activation, Con A did not result in B7 expression at any time point, as determined by both anti-B7 mAb staining and by expression of B7 mRNA as assessed by PCR. Thus, it appears that Con A directly activates B cells to express B7-2, but not B7. At no time was either B7 or the CTLA4 ligand expressed on T cells, even though it has been recently shown that activated human T cell clones can express B7. This data shows that the regulation of this new ligand is distinct from those events which regulate B7 expression. Whereas B7 expression occurs days after B cell activation, and only on a subset of the B cells,

B7-2 is up-regulated as soon as 6 hours after stimulation, with optimal levels achieved by 24 hours on as many as 75-80% of the splenic B cells. The early expression of B7-2 raises the possibility that it may play a critical role in triggering an immune response.

Example 4: *Anti-B7 mAb and hCTLA4Ig inhibit proliferation of a TH1 clone.*

A. T Cell Clone Proliferation Assays

Tumor cell transfectants were mitomycin-C (Sigma Chemical Co.) treated for 30-45 minutes and BALB/c spleen stimulators were irradiated for 2000 rads to prevent proliferation of stimulator cells in the assays. Following extensive washing in DMEM supplemented with 2.5% FCS, stimulators were added to individual wells of a 96 well microtiter plate at densities of 1 X 10^5 cells/well (transfectants) of 2.5 X 10^5 cells/well (irradiated spleen). The stimulators were preincubated with blocking or control antibodies for 15 minutes. PGL2 clones, which had been rested 7 days and treated with anti-l-Ad mAb (M5/114, ATCC) and complement (Accurate Chemical & Scientific Co, Westbury, NY), were then added at a density of 5 z 10^4 cells/well in the presence or absence of DOT. The 96 well plates were incubated at 37°C for 48 hours. Individual wells were pulsed with 1μCi [3H]thymidine/well and harvested 24 hours later. Counts are presented as mean CPM of triplicate wells. SE were $<10\%$.

B. Preparation of T Cell Clones and Tumors

$B7^+$ CHO cells were generated as described and maintained in DMEM media containing 10% FCS, 25μM HEPES, 2mM glutamine, 100 U. penicillin, 100 μg/ml streptomycin, 2mM NEAA, and 5 x 10^{-5} M 2ME (Complete Media) supplemented with .2mM proline, and 1μM methotrexate. EL-4 cells (obtained from ATCC) were transfected with MHC class 11 I-A^d cDNA subcloned into the pcEXV vector or co-transfected with murine B7 cDNA (kindly provided by Dr. Peter Linsley) subcloned into pcEXv and maintained in complete media supplemented with G418.

The Th1 clone, PGL2, was provided by Dr. Frank Fitch (University of Chicago, Chicago, IL), is specific for OVA peptide 323-339 and I-A^d restricted. Clones were maintained by bimonthly passage with irradiated BALB/cJ spleen in complete media and 400 μg/ml OVA (Sigma Chemical Co. St. Louis, MO. Clones were rested at least 7 days before use. A tryptic digest of OVA, DOT, was used as a source of peptide.

C. Results

We compared the ability of anti-B7 mAb and CTLA4Ig to inhibit the proliferation of an OVA-specific TH1 clone, PGL2, to either antigen-pulsed EL-4 cells expressing both I-A^d and B7 (ELAD-B7) or BALB/c (H-2^d) spleen cells. Peptide-pulsed ELAD/B7 cells (figure 4, panel A) and spleen cells (panel B) induced a strong proliferative response. Both hCTLA4Ig and the anti-B7 mAb (16-10A1) inhibited T cell activation to the ELAD-B7 cells, although the anti-B7 mAb was substantially more potent at the lower concentrations. The anti-B7 mAb, 16-10A1, inhibited proliferation > 70% at 10 μg/ml concentrations, while hCTLA4Ig inhibited < 20 % at the same concentration. This result most likely reflects the apparent higher affinity of this anti-B7 mAb for B7. The anti-mB7 mAb, 1G10, also inhibited the TH1 proliferative response, but with a similar dose response as that observed for CTLA4Ig, again consistent with the lower affinity of this mAb for B7. These results indicated that all three reagents bind to the functionally relevant epitope on the B7 molecule critical for CD28/B7 interaction and subsequent co-stimulation. In contrast, the proliferative response of the PGL2 clone, stimulated by peptide-pulsed BALB/c spleen cells was blocked only by hCTLA4Ig (figure 4, panel B). The anti-B7 mAbs, 16-10A1, and 1G10 were unable to block this response at concentrations that significantly inhibited the proliferative response to the B7 transfectants.

Example 5: *T cell activation in the allogeneic MLR depends on B7-2 ligation.*

A. Mixed Lymphoctye Reaction

Whole spleen (5×10^5) or enriched dendritic cells (5×10^4) were isolated from C57BL/6 mice, irradiated with 2000 rads, and incubated with blocking antibodies for 15 minutes prior to the addition of responders. As a source of T cells, 6×10^5 DBA/2J lymph node cells were added to cultures and incubated at 37°C with 5% $C0_2$ for three days. Individual wells were then pulsed with 1μCi/well [3H]-thymidine and harvested 12 to 16 hours later. Counts are represented as mean CPM of triplicate wells. SE were < 10%.

B. Results

Dendritic cells are the primary splenic subset responsible for initiating primary allogeneic T cell responses. Dendritic cells were prepared as described above. Flow cytometric analysis was performed as described above. We have shown (figure 2, panels I-L) that dendritic cells constitutively express both B7 and B7-2. We next addressed which CTLA4 ligand was critical for dendritic cell-initiated, T cell allogeneic responses.

First, as expected, enriched dendritic cells stimulated a potent T cell response. More importantly, T cell activation was inhibited by hCTLA4Ig and not by the anti-B7 mAbs (Figure 5). Even though the dendritic cell purity was not 100%, it is evident that the proliferation was in response to the dendritic cells and not contaminating B cells and macrophages since a greater proliferative response was observed using ten-fold less dendritic APCs than normal spleen cells. In fact, dendritic cell- and macrophage-depleted splenic cells were unable to induce proliferation above background levels, even at a 10-fold higher cell density. These data indicate that B7-2, expressed on dendritic cells, is a major cell adhesion/costimulation pathway in a primary allogeneic MLR.

Because numerous modifications and variations in the practice of the present invention are expected to occur to those skilled in the art, only such limitations as appear in the appended claims should be placed thereon.

REFERENCES CITED

The references listed below as well as all references cited in the specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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CLAIMS

1. An isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

2. The isolated and purified polynucleotide of Claim 1, wherein said polynucleotide is a DNA molecule.

3. The polynucleotide of Claim 1, wherein said encoded polypeptide is B7-2.

4. An isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, said polynucleotide preparable by a process comprising the steps of:

constructing a DNA library from a cell that expresses said polypeptide;

screening the library with a labelled nucleotide probe that encodes said polypeptide; and

selecting a clone that hybridizes to said probe.

5. An isolated and purified polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins,

and regulates T cell activation where the polynucleotide is preparable by a process comprising the following steps:

providing a library of cDNA from cells expressing the polypeptide;

transfecting cells with vectors comprising polynucleotides of the library;

panning the transfected cells by exposing the cells to an antibody to the polypeptide;

identifying those cells expressing the polypeptide; and

identifying the cDNA sequence comprising the polynucleotide that encodes the polypeptide of the present invention.

6. An isolated and purified polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

7. The polypeptide of Claim 5, wherein said polypeptide is B7-2.

8. The polypeptide of Claim 5, wherein said polypeptide is a recombinant polypeptide.

9. An expression vector comprising a polynucleotide that encodes a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation.

10. A process of preparing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation said process comprising:

transfecting a cell with a polynucleotide that encodes the polypeptide to produce a transformed host cell; and

maintaining the transformed host cell under biological conditions sufficient for expression of said polypeptide.

11. A process of screening a substance for its ability to interact with a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation, said process comprising the steps of:

providing a polypeptide, other than B7, that binds to CTLA4, or CD28, or CTLA4Ig, or homologous proteins, and regulates T cell activation:

testing the ability of said substance to interact with said peptide.

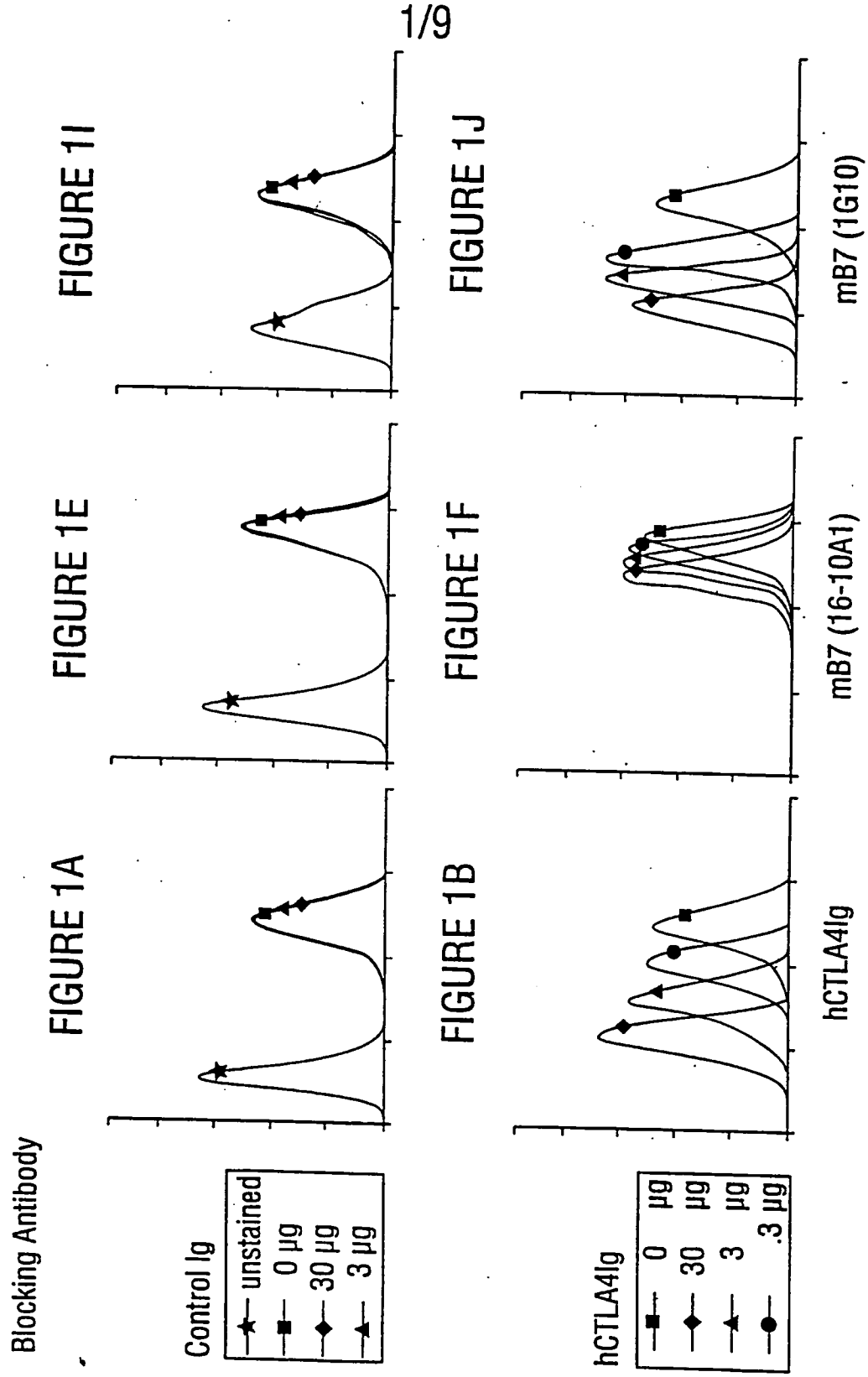


FIGURE 1A
FIGURE 1E
FIGURE 1I
FIGURE 1B
FIGURE 1F
FIGURE 1J
Blocking Antibody
Control Ig
unstained
0 µg
30 µg
3 µg
hCTLA4Ig
0 µg
30 µg
3 µg
.3 µg
hCTLA4Ig
mB7 (16-10A1)
mB7 (1G10)

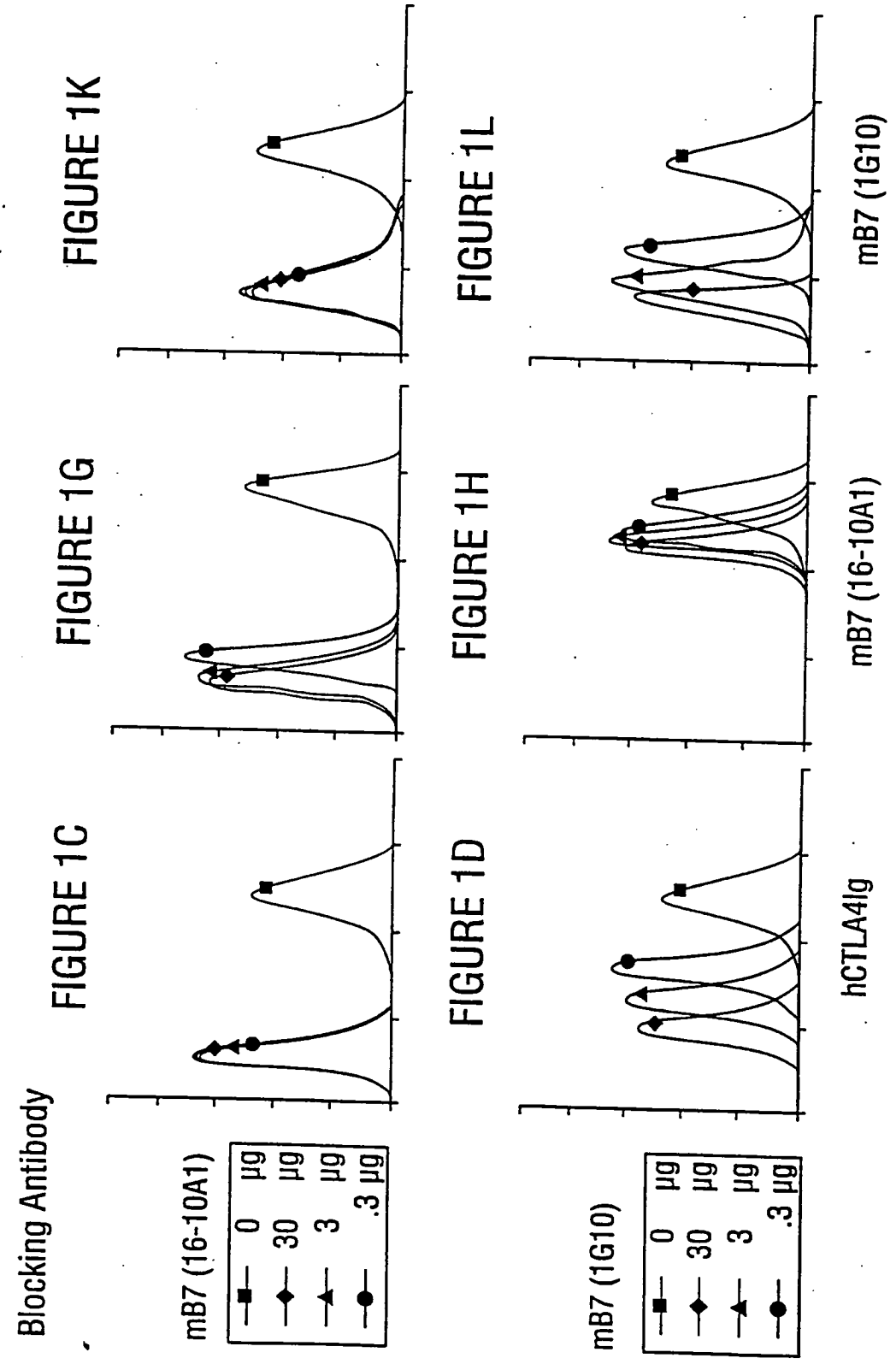
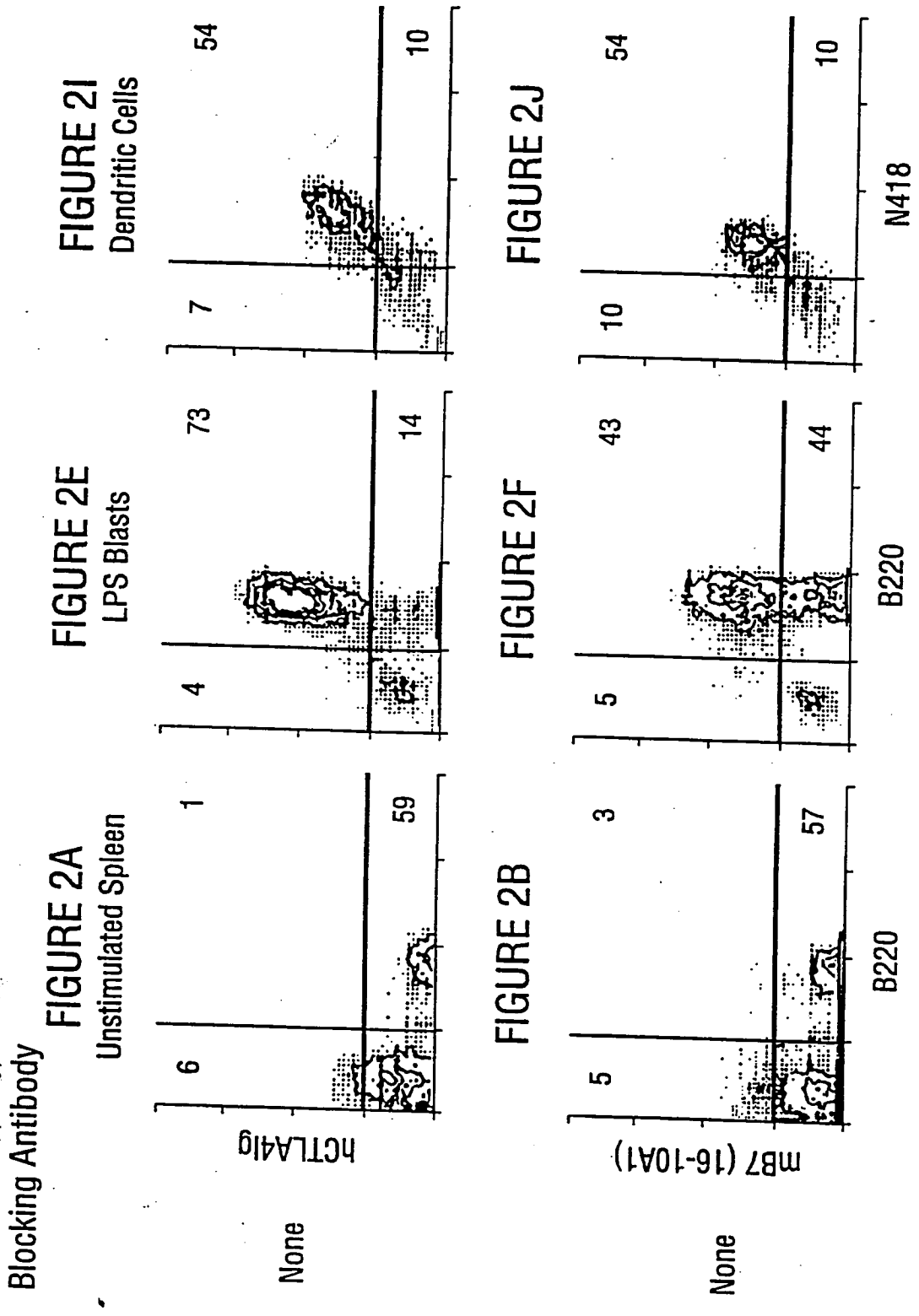
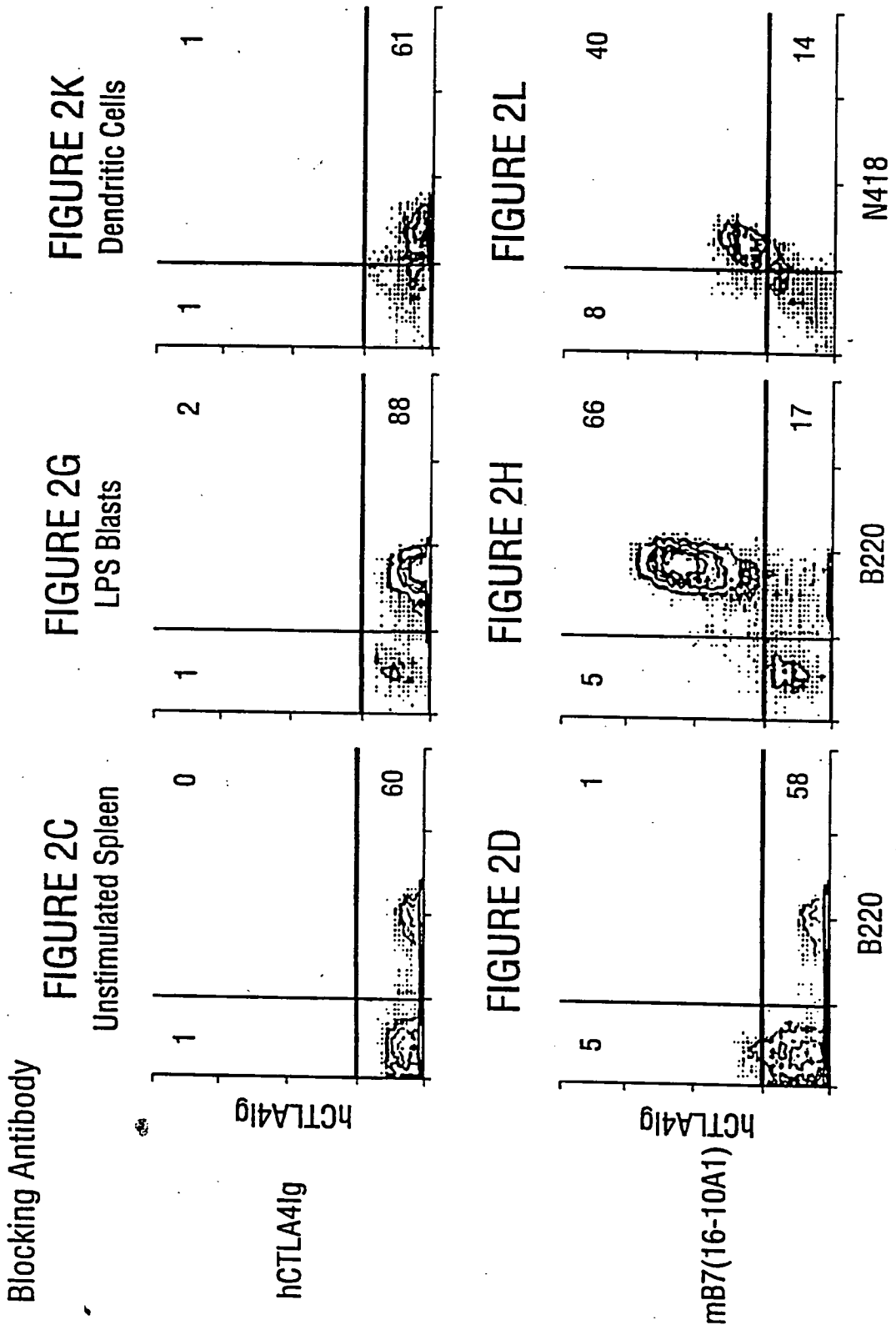


FIGURE 1K
FIGURE 1L
FIGURE 1G
FIGURE 1H
FIGURE 1C
FIGURE 1D
Blocking Antibody
mB7 (16-10A1)
0 µg
30 µg
3 µg
.3 µg
mB7 (1G10)
hCTLA4Ig
mB7 (16-10A1)
mB7 (1G10)


Blocking Antibody
None
hCTLA4Ig
FIGURE 2A
Unstimulated Spleen
6
1
59
FIGURE 2E
LPS Blasts
4
73
14
FIGURE 2I
Dendritic Cells
7
54
10
None
mB7 (16-10A1)
B220
FIGURE 2B
5
3
57
FIGURE 2F
5
43
44
FIGURE 2J
10
54
10
N418


Blocking Antibody
hCTLA4Ig
mB7(16-10A1)
hCTLA4Ig
B220
FIGURE 2C
Unstimulated Spleen
1
0
60
FIGURE 2G
LPS Blasts
1
2
88
FIGURE 2K
Dendritic Cells
1
1
61
FIGURE 2D
5
1
58
FIGURE 2H
5
66
17
FIGURE 2L
8
40
14
N418

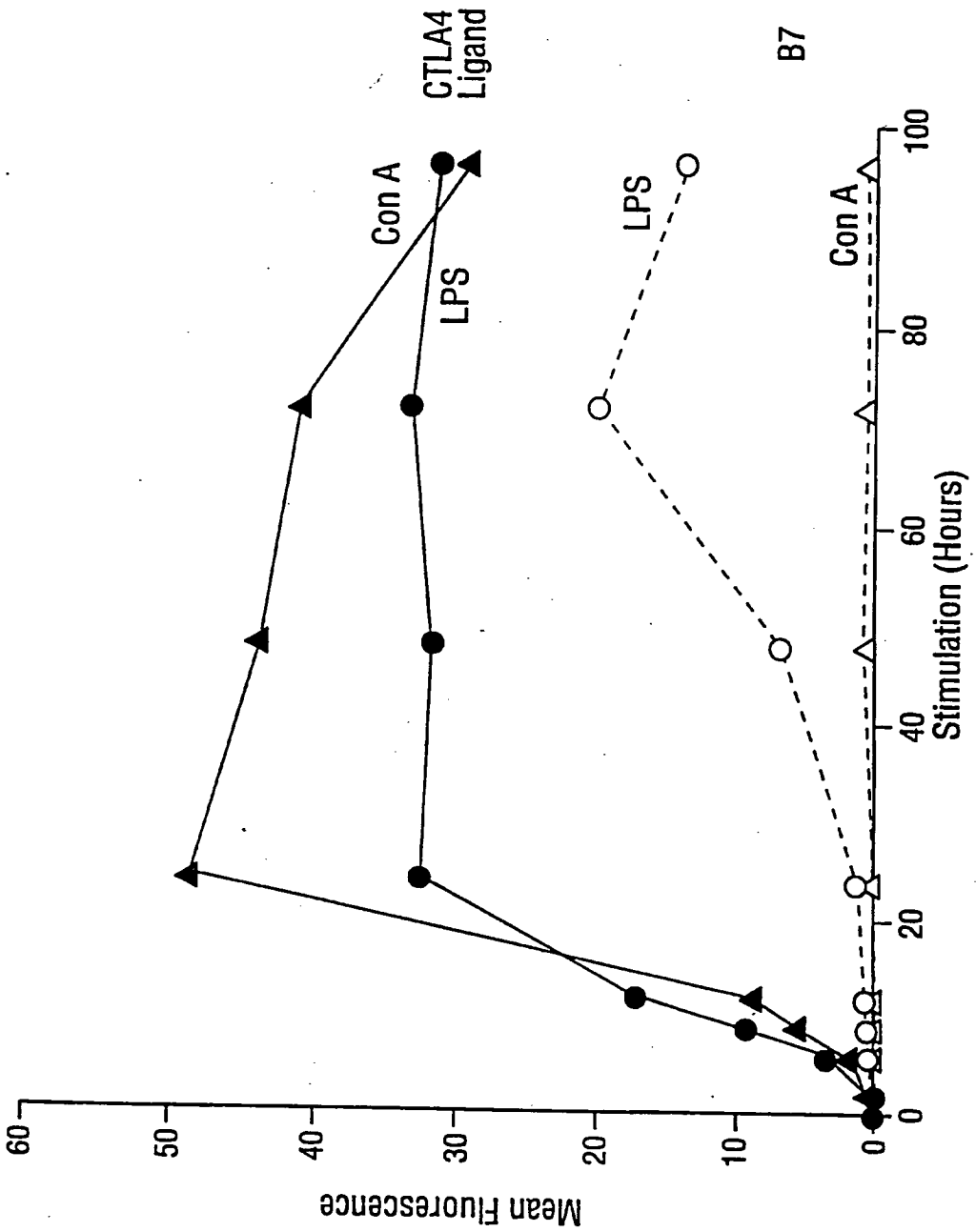

CTLA4 Ligand
B7
Con A
LPS
LPS
Con A
Mean Fluorescence
0
10
20
30
40
50
60
0
20
40
60
80
100
Stimulation (Hours)

FIGURE 3A

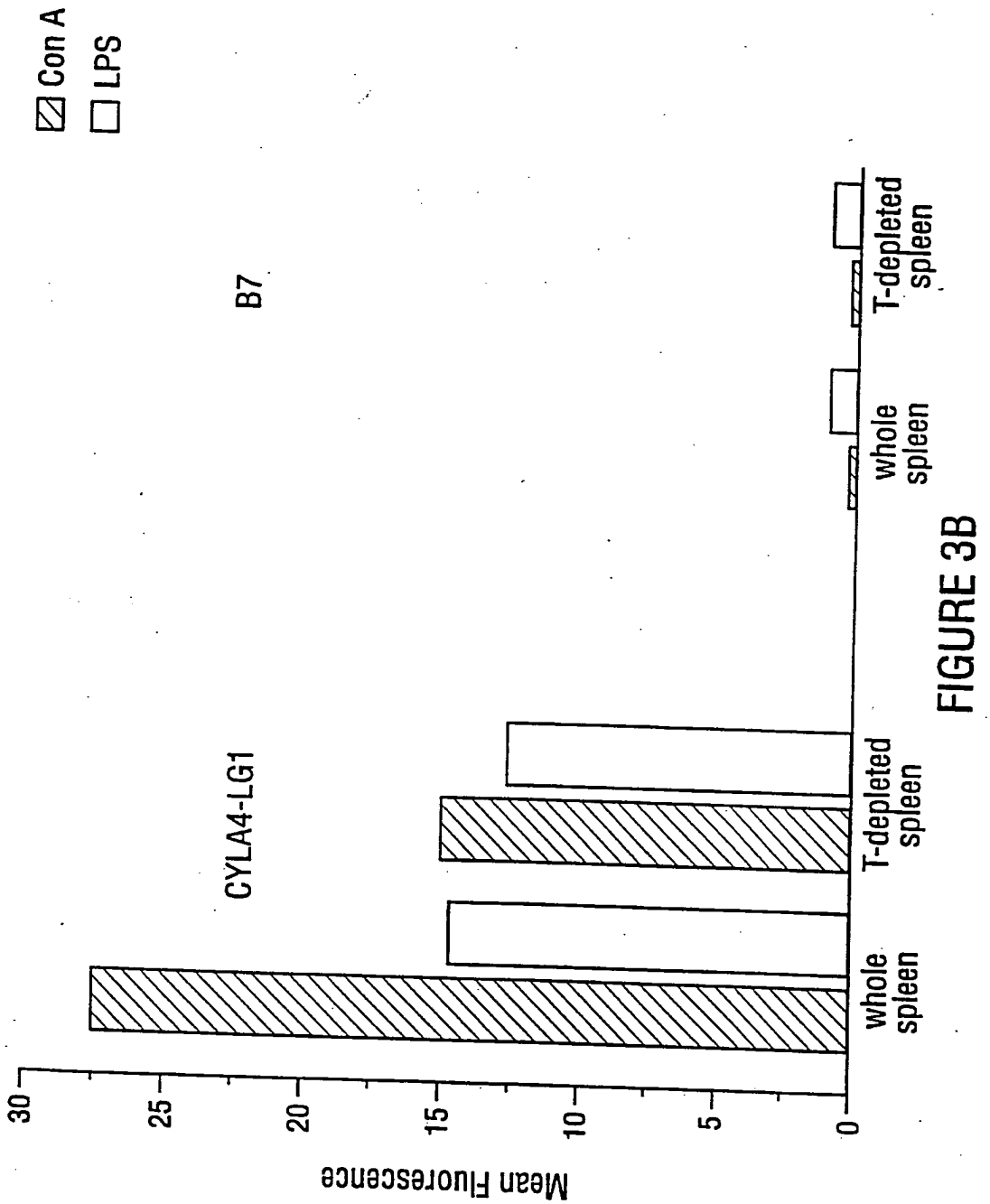

Con A
LPS
B7
CYLA4-LG1
Mean Fluorescence
0
5
10
15
20
25
30
whole spleen
T-depleted spleen
whole spleen
T-depleted spleen

FIGURE 3B

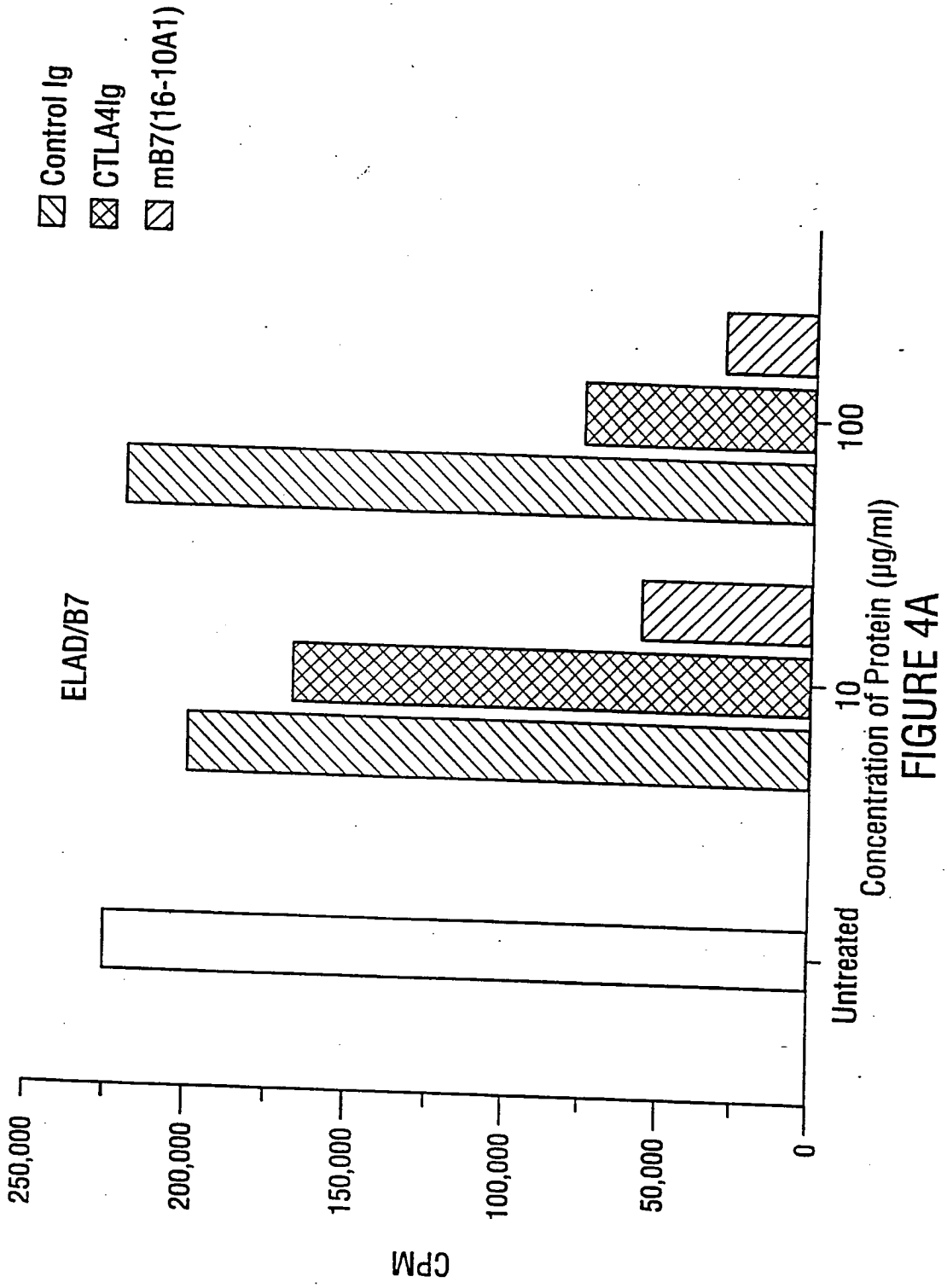

Control Ig
CTLA4Ig
mB7(16-10A1)
ELAD/B7
CPM
250,000
200,000
150,000
100,000
50,000
0
Untreated
10
100
Concentration of Protein (µg/ml)

FIGURE 4A

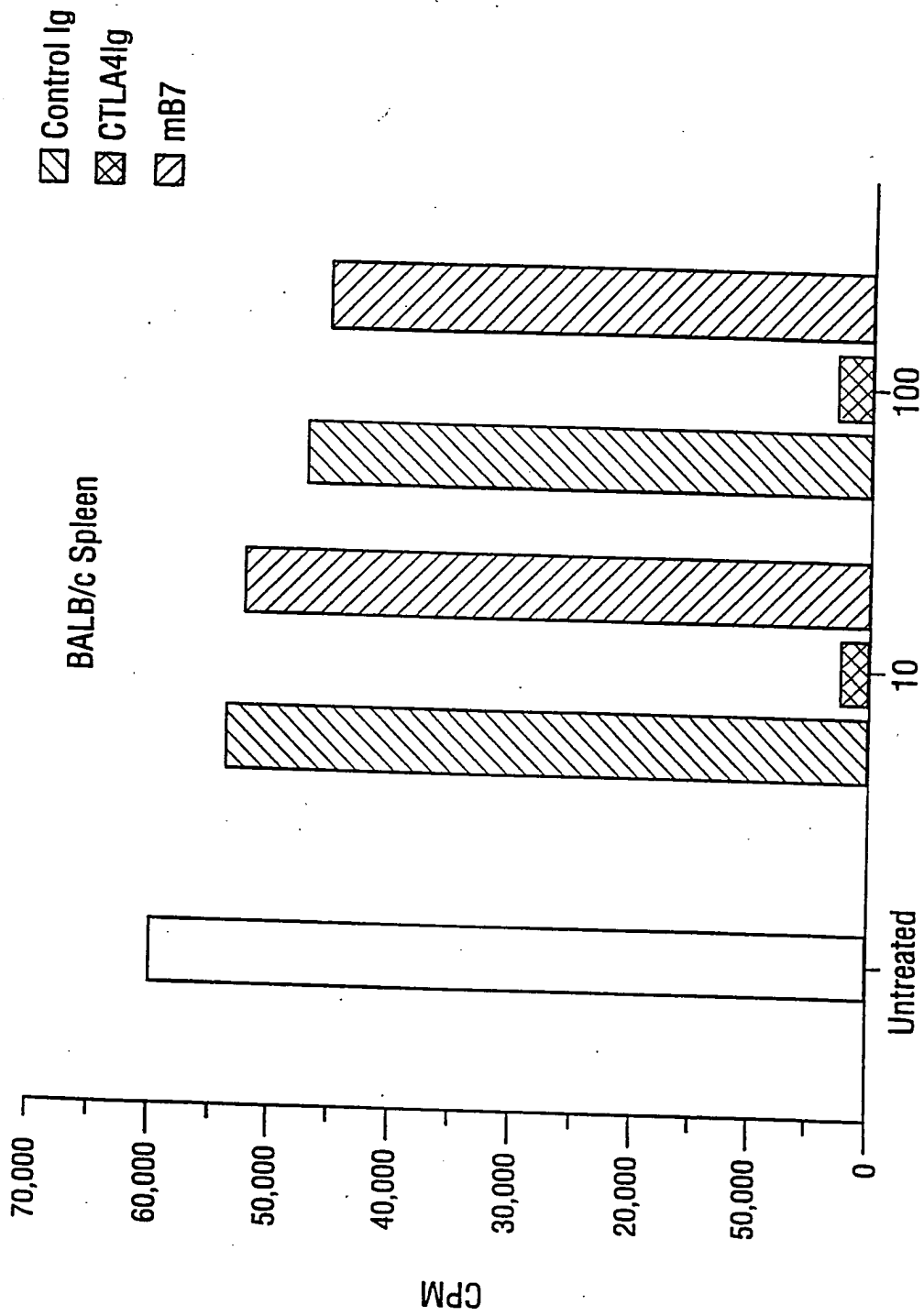

Control Ig
CTLA4Ig
mB7
BALB/c Spleen
CPM
70,000
60,000
50,000
40,000
30,000
20,000
50,000
0
Untreated
10
100
Concentration of Protein (µg/ml)

FIGURE 4B

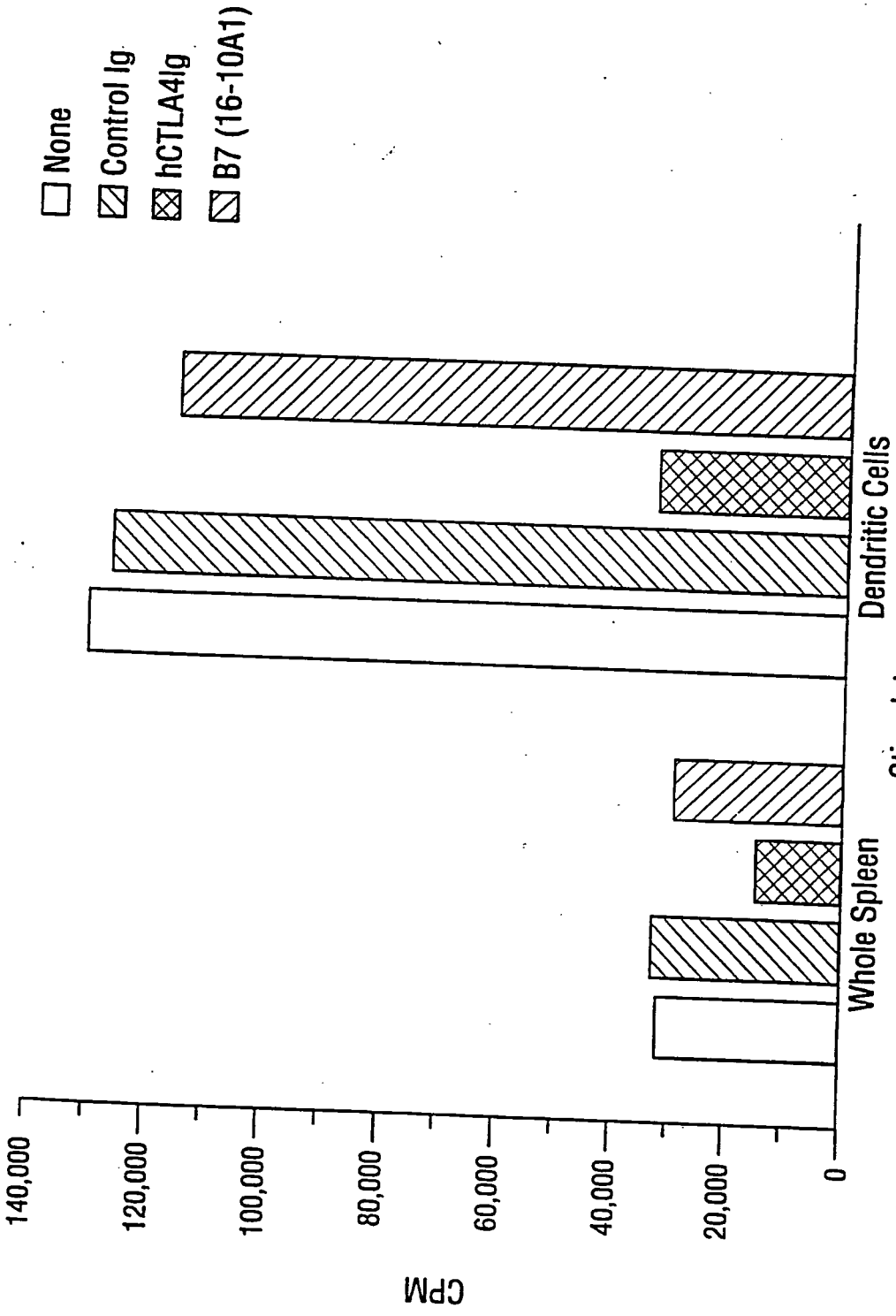

None
Control Ig
hCTLA4Ig
B7 (16-10A1)
Dendritic Cells
Stimulators
Whole Spleen
CPM
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0

FIGURE 5

INTERNATIONAL SEARCH REPORT

Internat' 'Application No
PCT/US 94/09252

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGY., vol.149, no.4, 15 August 1992, BALTIMORE US pages 1115 - 1123 AZUMA, M. ET AL.; 'Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line' see the whole document ---	1,2,6,8, 9,11
X	WO,A,93 00431 (BRISTOL MYERS SQUIBB) 7 January 1993 see the whole document ---	1,2,4-6, 8-11
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 1994	01 12 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 94/09252

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NATURE., vol.366, 4 November 1993, LONDON GB pages 76 - 79 AZUMA, M. ET AL.; 'B70 antigen is a second ligand for CTLA-4 and CD28' see the whole document	1-11
P,X	SCIENCE, vol.262, 5 November 1993, LANCASTER, PA FREEMAN, G.J. ET AL.; 'Cloning of B7-2 : a CTLA-4 counter receptor that costimulates human T cell proliferation."' see the whole document	1-11
P,X	IMMUNOLOGY TODAY, vol.15, no.7, 1 July 1994, CAMBRIDGE GB pages 321 - 332 JUNE, C.H. ET AL.; 'The B7 and CD28 receptor families.' see the whole document	1-11
E	EP,A,0 613 944 (BRISTOL-MYERS SQUIBB COMPANY) 7 September 1994 see the whole document	1,2,4-6, 8-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat' l Application No
PCT/US 94/09252

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300431	07-01-93	AU-A-	2240092	25-01-93
		CA-A-	2110518	07-01-93
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EP-A-0613944	07-09-94	AU-B-	5390194	28-07-94
		CA-A-	2113744	23-07-94
		FI-A-	940270	23-07-94